ISOLATION AND CHARACTERIZATION OF *Clostridium difficile* AND ITS TOXIN DETECTION FROM FAECAL SPECIMENS IN PATIENTS WITH ANTIBIOTIC ASSOCIATED DIARRHEA IN A TERTIARY CARE CENTRE

Dissertation submitted to

The Tamil Nadu Dr. M.G.R. Medical University

In partial fulfillment of the regulations

For the award of the degree of

M.D. MICROBIOLOGY

Branch- IV

DEPARTMENT OF MICROBIOLOGY
PSG INSTITUTE OF MEDICAL SCIENCES AND RESEARCH
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CERTIFICATE- I

This is to certify that the dissertation work entitled **"Isolation and characterization of *Clostridium difficile* and toxin detection from faecal specimens in patients with Antibiotic associated diarrhea in a tertiary care centre"** submitted by **Dr. Sneha May Kurian,** is work done by her during the period of study in this department from January 2016 to August 2017. This work was done under the guidance of **Dr. B. Appalaraju**, Professor and Head, Department of Microbiology, PSG IMS&R.

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CERTIFICATE- II

This is to certify that this dissertation work titled **"Isolation and characterization of *Clostridium difficile* and toxin detection from faecal specimens in patients with Antibiotic associated diarrhea in a tertiary care centre"** of the candidate **Dr. Sneha May Kurian** with registration number **201514402** is for the award of the degree **M.D. Microbiology, Branch IV.** I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **1**% percentage of plagiarism in the dissertation.

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ACKNOWLEDGEMENT

I take this opportunity to extend my gratitude to all those who helped me in completing this dissertation.

At the outset, I would like to thank my guide Dr. B. Appalaraju M.D, Professor and Head, Department of Microbiology, PSGIMS&R, Coimbatore for his advice and timely help in the preparation of this dissertation. His vast knowledge and zeal for learning has constantly motivated me to perform better.

I am thankful to all the Professors and Assistant Professors, Department of Microbiology, PSGIMS&R, Coimbatore who have gone out of their way to help me throughout my training. They have been a source of inspiration to me. I would also like to express my deepest gratitude to Dr. Marina Thomas, retired Professor, Department of Microbiology, PSGIMS&R, Coimbatore for her guidance and encouragement during the initial part of this study.

I would like to thank Dr. Ramalingam M.D., Dean, and the Research committee, PSGIMS&R, Coimbatore for choosing my dissertation to be funded with the PSG Prime intramural competitive funding.

I take this opportunity to convey my gratitude to Dr. Karthikeyan, Assistant Professor, Department of Community medicine, PSGIMS&R, Coimbatore for his help with the statistical analysis.

I thank my fellow post graduates and friends for their constant support throughout my study. I would like to thank all the technicians and staff for their assistance especially Mr. A.C. Baskaran, Mrs. Senthil and Mrs. Manonmani.

I am grateful to my family for being my pillar of strength. They have always stood by me and motivated me to achieve my goals. I thank them for their support, their patience and their prayers.

Lastly, I would like to thank God for his blessings and for being with me throughout this journey. Without Him, nothing would have been possible.

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INTRODUCTION

Clostridium difficile (*C. difficile*) is a Gram positive, spore forming bacillus which grows anaerobically. It is a part of the normal flora in the gastrointestinal tract and is seen in 1-3% of healthy adults with a higher rate of colonization in infants. However, there is an increase in the incidence following long term hospitalization, surgery and more importantly following antibiotic usage. [(1)] Disruption of the normal flora occurs following antibiotic usage thereby allowing endogenous and environmental *C. difficile* to proliferate in the colon and produce toxins leading to *Clostridium difficile* infection (CDI).[(2)] Infection can result in a wide spectrum of diseases ranging from uncomplicated diarrhea to pseudomembranous colitis and toxic megacolon and can even lead to death. [(3)] In India, the estimated incidence of *Clostridium difficile* associated diarrhea (CDAD) has been found to be between 15- 25% in paediatric and adult patients who are on antibiotics.[(4)] Pépin et al. reported that fluoroquinolones were the most common antibiotics associated with the emergence of *C. difficile* followed by cephalosporins, macrolides, clindamycin and intravenous β–lactams/β-lactamase inhibitors. [(5)]

Pathogenesis of CDI is primarily attributed to two toxins produced by the bacteria- an enterotoxin (TcdA) and a cytotoxin (TcdB). These are encoded by the genes *tcdA* and *tcdB* respectively. [(6)] In addition to this, some strains contain a potential virulence factor named the binary toxin (CDT) encoded by *cdtA*

(binding component) and *cdtB* (enzymic component). [7] Toxin detection therefore is essential in confirming CDI.

Diagnosis is based on a combination of factors including the patients's symptoms, stool tests and rarely endoscopic and radiological findings. The various tests that can be done include a stool culture, cell cytotoxicity assay, enzyme immunoassay (EIA), polymerase chain reaction (PCR) and stool glutamate dehydrogenase (GDH) assay. [8] No gold standard exists for the diagnosis of CDI. Stool culture on selective cycloserine cefoxitin fructose agar (CCFA) is considered to be the most sensitive method for detection however specificity is low as asymptomatic carriage of *Clostridium difficile* is high among hospitalized patients. Therefore to increase specificity, the isolates can be further tested for toxin production by cell cytotoxicity assays or enzyme immunoassays. However, culture and cell cytotoxicity assays are much more time consuming compared to EIAs. [9] As EIAs are rapid, easy to perform and inexpensive; these are preferred as the method of choice for screening stool samples. GDH is a cell associated enzyme antigen found on most isolates of *C. difficile.* Its detection in stool samples is therefore a sensitive screening test for *C. difficile.* A two-step approach is usually followed where GDH antigen is first detected by EIA and GDH positive samples are then tested for toxins by toxin EIAs or molecular testing for toxin genes. GDH assays however have a lower sensitivity of 87.6% when compared to toxigenic culture. [10] PCR therefore offers a third step by which isolates can be tested to see if they are potentially toxin producing isolates. [11] PCR is found to have better sensitivities

than anaerobic culture methods and cytotoxicity assays. Recently, nucleic acid amplification techniques have also been developed for diagnosis. [12]

Mild to moderate cases of *C. difficile* can be treated with oral metronidazole but if no response is noted, vancomycin can be initiated in the patient. In severe cases, oral vancomycin is the drug of choice with some patients even requiring emergent colectomy.[13]

Since *Clostridium difficile* is one of the most common causes of nosocomial diarrhea, it is necessary to understand the incidence of CDI and the risk factors associated with it so that effective measures can be taken by hospitals to prevent transmission. Sensitive and rapid tests are the need of the hour. This will help us in early diagnosis, treatment, and management of the patient and in understanding how to judiciously use antibiotics.

AIM & OBJECTIVES

AIM

To study the incidence of *Clostridium difficile* infection in patients with antibiotic associated diarrhea and compare rapid assay for diagnosis with culture and molecular methods.

OBJECTIVES:

1. To isolate *Clostridium difficile* from faecal samples of patients with antibiotic associated diarrhea who are suspected to have Clostridium difficile infection at our hospital.
2. To compare rapid enzyme immunoassays against culture and PCR in the diagnosis of *Clostridium difficile* infection.
3. To determine the risk factors and antibiotics commonly associated with *Clostridium difficile* infection.

REVIEW OF LITERATURE

HISTORICAL PERSPECTIVES:

Pseudomembranous colitis (PMC) was first discovered way back in 1893 by Finney, but the causative agent for the disease was not known then. Finney noticed plaque like membranes in the stomach and small bowel of his patient Mary G., a 22 year old post-operative female who was being treated by William Osler, and termed it "diphtheritic colitis". Initially, *Staphylococcus aureus* was thought to be the causative agent of the disease as it was isolated in stool of patients presenting with high fever and abdominal distress. Oral vancomycin therefore became the standard treatment for this disease. But several of the patients in whom it was isolated, showed no intestinal lesions on autopsy.[14, 15]

In 1935, Hall and O' Toole first described the presence of an anaerobic bacteria as a part of the intestinal flora of healthy, newborn infants. [16] The bacteria was initially named "*Bacillus difficilis"* owing to the difficulty in isolating it and its slow growth in culture. Although initially considered nonpathogenic, 40 years later, researchers discovered its pathogenic potential. The name was changed to *Clostridium difficile* and the ability of the organism to produce toxins was noted. This name was derived from the Greek word "Kloster" meaning spindle, a reference to its appearance on Gram staining. [1]

Larson *et al.*, in 1977, demonstrated the presence of a bacterial toxin from the faeces of a young girl who developed pseudomembranous colitis

following treatment for acute pharyngitis with oral penicillin. This toxin was shown to produce characteristic cytopathic effects on cultured cell lines but a specific microorganism was not implicated. The toxin could be neutralized by *Clostridium sordellii (C. sordellii)* antitoxin leading to the premature conclusion that *Clostridium sordellii* was the causative agent for antibiotic associated pseudomembranous colitis. However, the organism could not be isolated from the faeces of patients with the disease. Repeated cultures from patients with confirmed pseudomembranous colitis showed the recovery of clindamycin resistant clostridial isolates. This organism was identified biochemically and culturally to be *C. difficile*. It was found to produce a toxin which could be neutralized by *C. sordellii* antitoxin and thus the connection between *C. difficile* and pseudomembranous colitis was made. [14]

There was increasing speculation that antibiotic usage might be a risk factor for the development of PMC. This was first studied by Tedesco *et al*. in 1978, who noted that 21% of patients on clindamycin developed the disease. This was also the first study where endoscopy was used as a routine diagnostic procedure in patients with antibiotic associated diarrhea. [14, 15]

Bartlett and Chang *et al*., in early studies, used the hamster model for diagnosis of *C. difficile*, demonstrating its cell cytotoxicity effects, detecting toxin B, confirming the role of antibiotics in inducing PMC and to demonstrate the effect of oral vancomycin in the treatment of the disease. [4, 15] Although clindamycin was the first antibiotic found to be associated with antibiotic associated diarrhea, studies in the 1980s showed that cephalosporins, broad

spectrum penicillins, amoxicillin were equally responsible for inducing CDI. The role of fluoroquinolones in causing CDI was reported much later. Antineoplastic drugs like methotrexate were also recognized as drugs capable of inducing pseudomembranous colitis. [15]

The first test that was developed was the cytotoxin neutralization test by utilizing *C. sordellii* or *C. difficile* antitoxin. However, due to the long turnaround time, other methods were developed such as latex agglutination tests, dot immunoblot assays, PCR, stool culture on selective media and EIAs. EIAs came into use in the 1980s, but the sensitivity for toxin detection was found to be around 75% when compared with the cell cytotoxicity assays. Hence, a screening test was devised wherein the EIA detected glutamate dehydrogenase which was found to have a sensitivity and specificity approaching nearly 100%. [15]

Early clinical trials were highly successful in proving the effectiveness of vancomycin in the treatment of CDI. Subsequent studies showed that metronidazole was equally effective. Due to the reduced cost of the drug and concerns that vancomycin usage could promote emergence of vancomycin resistant enterococci strains, metronidazole became the drug of choice for treating CDI. [15]

MILESTONES:

- 1893- First reported case of pseudomembranous colitis by Finney
- 1935- Discovery of *Bacillus difficilis* by Hall and O' Toole in the gut of healthy infants
- 1970- *Bacillus difficilis* renamed to *Clostridium difficile*
- 1977- Demonstration of bacterial toxin in the faeces of a patient diagnosed with PMC by Larson *et al.*
- 1978- *Clostridium difficile* proved to be the causative agent of PMC by Bartlett *et al.*
- 1978- Tedesco *et al.* confirmed the association between clindamycin usage and the development of the disease
- 1998- 2004- Large outbreak of CDI in Québec with an overall mortality of 6.9% [(1)]
- 2005- Emergence of a new, highly toxic strain of *C. difficile* was reported in North America and designated B1/NAP1/027 [(1)]

PHYLOGENY AND NOMENCLATURE:

The *Clostridium* group represents an ancient prokaryotic lineage. It diverged from the bacterial domain more than a billion years ago. This was around the time when molecular levels of oxygen in the atmosphere began to increase. It is estimated to be older than *Escherichia, Campylobacter,* and

Helicobacter groups. It has been described under the phylum *Firmicutes.* The class *Clostridia* encompasses a group of obligate, Gram positive anaerobes with endospore formation. Initial classification of *Clostridia* was based on these phenotypic characteristics. However, 16S rRNA sequencing showed that the *Clostridia* were phylogenetically incoherent. [17]

In a landmark study by Collins *et al.* in 1994, he proposed that *C. difficile* belongs to cluster XI which is more closely related to the non-spore forming species *Peptostreptococcus anaerobius* and *Eubacterium tenue* than the type species of *Clostridia, Clostridium butyricum.* By this scheme, *C. difficile* does not cluster with many other familiar clostridial species such as *C. perfringens, C. tetani* and *C. botulinum.* This distance is manifested in the sporulation gene patterns. *C. difficile* genomes lack genes such as *spoIVFB, cotS, bofA, cotM, yhdD, gerA* and *gerC,* which are widespread among other clostridial species. But *C. difficile* genome encodes certain proteins that are not found in other clostridia. [17, 18]

Bergey's Manual of Systematic Bacteriology now places *C. difficile* under *Peptostreptococcaceae* along with a number of other *Clostridium* species. In 2013, further taxonomic revision was called for, with a proposed name change from *Clostridium* to *Peptoclostridium difficile.* But it is highly unlikely that this name change will be followed. [17]

MORPHOLOGY:

Clostridium difficile are straight, Gram positive, motile rods which are seen singly and measure about 0.5- 1.9μm × 3.0- 16.9μm. They have oval, subterminal/ terminal, non-bulging/ bulging spores. Occasionally at 48 hrs, bipolar spores may be seen. Spores are generally wider than the vegetative body. Due to its subterminal position, it gives the bacteria a club shaped appearance. Spores are easily visualized by Gram staining or by phase contrast illumination of a wet film. Special staining methods are not required. Irregularity in staining is noted in cultures more than a day or two old. In early spore formation, the spore stains intensely. Following spore maturation, on staining it shows a colourless centre with a peripherally stained ring. [19]

Wet films from Robertson's cooked meat broth show characteristic oscillatory movement. Motility is due to the presence of peritrichous flagella. Capsular material has been shown to be present on the surface of *C. difficile.* Diaminopimelic acid provides the cross link in the peptidoglycan layer. Plasmids have also been found which help in strain identification. [19]

GENETICS:

Genes encoding the clostridial toxins are located on the chromosome. The toxins have enterotoxic and cytotoxic activities and have been labelled toxin A and toxin B respectively. Analysis of toxin genes can be demonstrated by gene probes, pulsed field gel electrophoresis and other molecular approaches. These genes are frequently associated with unstable genetic

elements such as plasmids, transposons, and bacteriophages. This often results in phenotypic properties, such as genetic instability and the capacity of toxigenicity to be dispersed by horizontal gene transfer to other microbial species. [19]

Sullivan *et al*. separated the two toxins and purified them by DEAE ion exchange chromatography and observed molecular masses of 440‑500 kDa for toxin A and 360‑470 kDa for toxin B. Banno *et al*. purified the corresponding toxins to homogeneity as determined by polyacrylamide gel electrophoresis (PAGE) analysis using gel filtration and ion-exchange chromatography. Their molecular masses were 550‑600 kDa and 450‑500 kDa, respectively. Each of the two toxins was converted to an apparent single, smaller molecular form, 190‑200 kDa, determined by SDS-PAGE analysis after heating at 100°C for 5 min in the presence of SDS and 2-mercaptoethanol. The toxins were found to be immunologically differentiable by cross-neutralization studies in cytotoxicity and mouse lethal assays. Cross-reactivity without neutralization between the toxins and corresponding heterologous monoclonal antibodies have, however, been observed, thus indicating common epitopes. However, it was shown that toxin A nonspecifically bound certain monoclonal antibodies and that this nonspecific binding occurred through the carbohydrates on the Fab component. [19]

HABITAT:

Clostridium difficile is a part of the normal intestinal flora in 1-3% of healthy adults and 15-20% of healthy infants. It is therefore excreted in the faeces of patients as well as carriers. Transmission of the organism is via the feco- oral route. CDI can be acquired from an endogenous or exogenous source. Exogenous sources include inanimate objects or surfaces in the environment which are contaminated with faeces containing *C. difficile* spores. It can also be transmitted by health care workers who come in contact with infected patients or infected faeces containing the spores. Therefore the rates of colonization considerably increase following long term hospitalization and surgery. Infection is prevalent in hospitals and nursing homes where patients frequently receive antibiotics. Following ingestion of the spores, the spores reach the stomach where it is able to survive the acidic pH and then reaches the small intestines. Usage of antibiotics destroys the intestinal microflora, thereby enabling the proliferation of *C. difficile* in the colon with production of toxins. Incubation period is usually 7 days following exposure but can extend upto 4 weeks. The infected patient can either be an asymptomatic carrier or present with symptoms ranging from diarrhea to colitis and in severe cases it can lead to death of the patient. [1, 2, 4]

CULTURE MEDIA USED FOR ISOLATION OF *Clostridium difficile:*

Several procedures have been described for the isolation of *C. difficile.* Isolation of the organism from stool specimens requires usage of a selective media or spore selection techniques or a combination of both methods.

Transport media are not required. Liquid stool samples can be directly plated on the media. Solid samples can be diluted 1:1 using buffered gelatin as a diluent. As *C. difficile* is an anaerobic organism, the inoculated media needs to be incubated anaerobically at 37°C for 48hrs. [20]

Selective solid media available:

1) Cycloserine cefoxitin fructose agar (CCFA): This is the media of choice for isolating the organism. It is a selective and differential medium. The original medium as described by George W L *et al.* contains cycloserine (500µg/ml), cefoxitin (16µg/ml), fructose and 50% egg yolk suspension. In addition to this, it contains proteose peptone, Na_2HPO_4, KH_2PO_4, NaCl, $MgSO_4$, agar and 1% neutral red solution as the indicator. *C. difficile* produces yellow colonies with ground glass appearance on this medium. Yellow- green fluorescence (chartreuse) is observed under ultraviolet light. This medium has currently been modified by various commercial manufacturers resulting in variations in the ability of the media in isolation of the organism. Changes include variations in the concentration of the antibiotics and replacement of egg yolk with blood.[20-22]
2) Clostrisel agar
3) Egg yolk- Neomycin agar (EYA): Neomycin (100µg/ml) is added to egg yolk agar base and autoclaved. [21]

4) Reinforced clostridial agar with cresol: Prepared by adding *p*- cresol at a final concentration of 0.2% to reinforced clostridial agar (BBL) before pouring the plates. [21]
5) ChromID *C. difficile* agar (bioMérieux): Black coloured colonies are produced due to the chromogen that is incorporated. [23]
6) Tryptone soy agar with 5% sheep blood

Non selective solid media available:

1) Brucella agar supplemented with hemin, Vitamin K_1 and 5% sheep blood [21]
2) Brain heart infusion agar [23]
3) Anaerobic blood agar – Non hemolytic, large grey colonies are seen with characteristic horse stable odour. [24]

Selective liquid media available:

Broths are commonly supplemented with 1% neutral red as a pH indicator

1) Cycloserine cefoxitin fructose broth is commonly used in environmental sampling[23]
2) Cycloserine cefoxitin mannitol broth supplemented with taurocholate and lysozyme increases the sensitivity and can recover even as low as 10cfu/ ml of *C. difficile.* [23]

Non selective liquid media available:

1) Thioglycollate broth supplemented with peptic digest of blood, hemin and Vitamin K_1 [21]
2) Brain heart infusion broth (BHIS) supplemented with yeast extract (5mg/ml) and 0.1% cysteine [23]
3) Robertson's cooked meat broth (RCM): Solid meat particles facilitate growth of the organism and reducing substances lower the oxidation-reduction potential. [24]

Spore selection techniques:

1) Heat shock method (HS): 2-4 ml of stool is heated to 80°C in a water bath for 10 minutes and 1 drop is subsequently plated on the media.
2) Ethanol treatment (ET): Equal amounts of stool and absolute ethanol are mixed for 30 minutes and 1 drop is plated onto the media. [20]

Ethanol treatment is considered to be better than heat shock treatment for inducing sporulation. But there is no difference in sensitivity between direct plating onto a selective media and the ET method. [20]

BIOCHEMICALS:

Biochemical reactions reveal the physiological capabilities of the organism (Table 1).

Table 1: Biochemical reactions observed with *C. difficile* [24, 25]

Biochemical test	**Reactions with *C. difficile***
Catalase	Negative
Kanamycin (1mg)	Sensitive
Vancomycin (5mg)	Sensitive
Colistin (10mg)	Resistant
Spot indole	Negative
Lecithinase	Negative
Lipase	Negative
Glucose	Fermented
Lactose	Not fermented
Mannitol	Fermented
Fructose	Fermented
Sucrose	Not fermented
Xylose	Not fermented
Maltose	Not fermented
Aesculin hydrolysis	Variable (+/-)
Nitrate reduction	Negative
Reverse CAMP	Negative

An important adjunct to biochemical tests is the demonstration of metabolic end products through gas liquid chromatography (GLC). This is achieved following culture in anaerobe identification medium with glucose (AIMG medium). *C. difficile* converts tyrosine to *p*- cresol which can be detected by GLC. [20] Other fatty acids produced include large amounts of acetic acid and iso- butyric acid. Minor amounts of butyric, iso- valeric, valeric, iso- caproic, caproic and propionic acid are also produced. [25]

VIRULENCE FACTORS AND PATHOGENESIS:

The virulence mechanism of *Clostridium difficile* is a three step process beginning with disruption of the colonic microbiota, followed by adhesion of the bacteria to the host cells and subsequently multiplication of the bacteria. Toxin production occurs in the last phase. Emergence of epidemic strains of *C. difficile* such as B1/NAP1/027 suggest that there are factors that stimulate sporulation/ germination, adhesion and persistence of the organism in the gut. [26, 27]

Sporulation And Germination Of *C. Difficile*:

Transmission of *C. difficile* is by the feco- oral route. Spores are the infectious vehicle as the vegetative forms are unable to survive outside the host or in the acidic environment of the stomach. Spores are highly resistant and metabolically dormant forms which persist for long periods in the environment. They are resistant to some disinfectants such as 70% isopropyl alcohol, low levels of chlorine and show mixed results when subjected to disinfection with

hydrogen peroxide. [28] They are also resistant to commonly used antibiotics such as clindamycin, cephalosporins and fluoroquinolones. [29] Following ingestion of the spores, they germinate in the gut to produce vegetative cells that produce toxins. Sporulation occurs in response to stress and germination in response to specific bile salts in the small intestine and start producing toxins which initiate the disease. Multiple studies have suggested that epidemic strains such as ribotype 027 tend to produce more spores in vitro. However, it has not yet been established if the same findings are seen in vivo as well. Paredes *et al.* compared the sporulation signaling pathways in *Bacillus spp.* and clostridia. The analysis revealed that almost all the regulators and signaling molecules downstream of the master regulator of sporulation (SpoOA) were conserved but the signaling molecules upstream of SpoOA which contribute to SpoOA phosphorylation and activation were absent in clostridial genomes. Therefore, factors which influence sporulation remain unknown.[26, 30, 31]

Factors that affect germination are also not very clear. Parades- Sabja *et al.* found that for germination to occur, a small molecule (germinant) needs to bind to a receptor (CspC) which is present on the spore's inner membrane. The germinants for *C. difficile* have been identified as the bile acid taurocholate and the amino acid glycine. A proteolytic cascade is then activated that leads to degradation of the spore peptidoglycan, release of calcium dipicolinic acid, rehydration of the spore and ultimately outgrowth of the cells. Germination is inhibited by secondary bile acids in the colon. Therefore, therapeutic agents

which block the action of the germinants would have application in prevention of *C. difficile* infection. [26, 31]

Colonisation of the Gut Mucosa:

Although gut pathologies are produced by the toxins, a prerequirement for the establishment of CDI is the clearance of gut microbiota by antibiotics. In the colon, sialidase-producing commensal bacteria cleave sugars from glycosylated proteins that are bound to the epithelial cell membrane, which releases free sialic acid into the lumen. Primary fermenters break down complex carbohydrates into short-chain fatty acids. Both of these metabolites are rapidly consumed as energy sources by commensal bacteria. However, antibiotic treatment can deplete competing commensal bacteria, which leads to an abundance of sialic acid and succinate, a short-chain fatty acid that is produced during fermentation. *C. difficile* has genes for both sialic acid catabolism and succinate transporters, which enables it to use the excess sialic acid and succinate for growth. Colonisation of the gut mucosa by *C. difficile* is therefore the first step in development of CDI. In a normal individual, one gram of faeces contains upto 10^{12} bacteria which resist colonization and multiplication by *C. difficile.* Lactobacilli and Group D enterococci display the most antagonistic activity. Antibiotic induced shift in the gut mucosa creates an environment that is conducive for *Clostridium difficile* infection. The expression of cell surface proteins and surface layer proteins are also stimulated by antibiotics such as ampicillin and clindamycin. These proteins increase adherence to the colonic epithelial cells. Mutations in genes encoding

these proteins cause an attenuation in the virulence of the bacterium. Mucolytic enzymes are secreted by the bacteria, such as cell surface protein Cwp84, which degrade the colonic mucosa. [31- 33]

Virulence Factors:

Virulence factors which play a role in the pathogenesis of CDI can be classified as follows: [26, 27]

1) Toxins- Toxigenic strains are the only ones which can cause infection. The different toxins associated with *C. difficile* are Toxin A, Toxin B and the Binary toxins.
2) Non toxin virulence factors- Non toxin virulence factors play a role in bacterial colonization. The factors include bile tolerance, cell wall proteins and the flagella.

Toxins:

Following ingestion of spores, they germinate into a vegetative morphotype which produces toxins in case a toxigenic strain is involved. The toxins are endocytosed by the colonic epithelial cells, damage the actin cytoskeleton and lead to cell death. [26]

Toxin A and Toxin B:

Toxigenic *C. difficile* produces one or two glucosyltransferase toxins called Toxin A (enterotoxin) and Toxin B (cytotoxin) which are encoded by TcdA and TcdB respectively. This terminology originated from the observed

actions of these toxins- demonstration of fluid accumulation in intestinal loop models and the cytopathic effects on tissue culture monolayers, respectively.[30]

Toxin A disrupts adherence of colonic mucosal cells to the basement membrane and damages the villous tips. It causes necrosis, increased intestinal permeability and inhibition of protein synthesis. It also affects prostaglandin A2, thereby producing prostaglandins and leukotrienes. A viscous, bloody fluid is produced in response to this tissue damage. Toxin B enters the cells by endocytosis and causes cell death. There is no considerable enterotoxic activity and it exerts its effect once the gut mucosa has been damaged. Toxin B is 1000 times more potent than Toxin A. [30]

The genes encoding the toxins are located within a 19.6kb genomic island known as Pathogenecity Locus (PaLoc).The toxins have a receptor binding domain, a transmembrane domain and a glucosyl transferase domain (Fig. 1). Hoffman *et al*. noted that the enzyme and cytotoxic activity of toxin A and B was to be found at the toxin's N-terminus. The middle section of both toxins includes a transmembrane domain, which is thought to encode for the translocation of the toxin into the cytosol. The C-terminal of the toxin encompasses the receptor-binding domain and is constructed of repetitive peptide elements. [30]

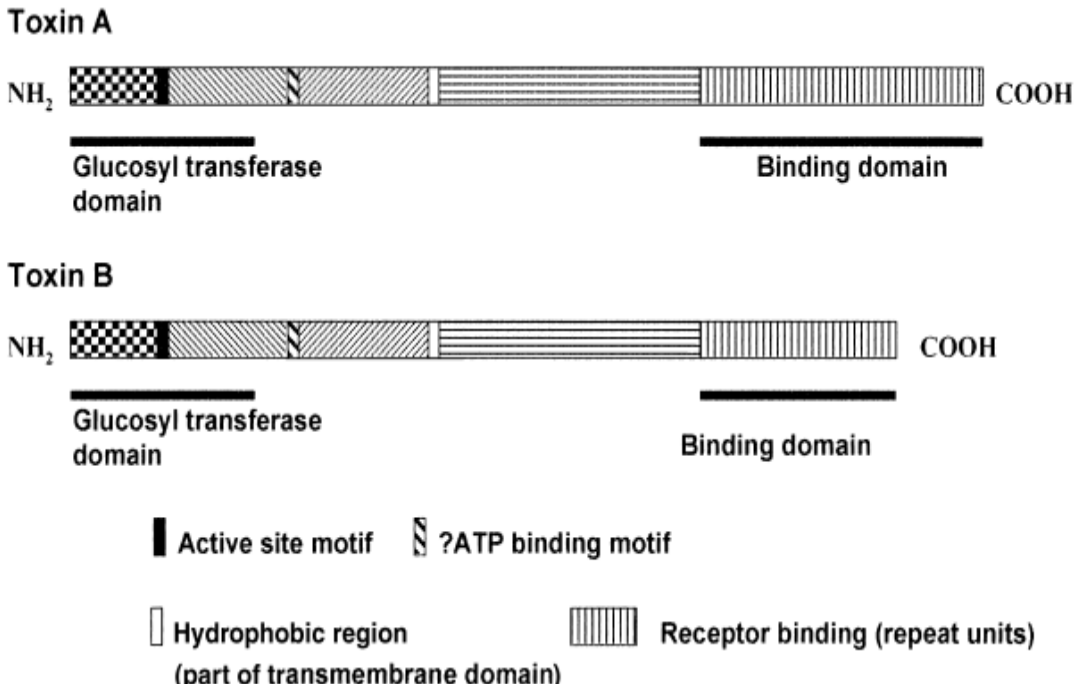


Figure 1: Structure of Toxins A and B (from ref. 30)

The carboxy terminal of toxin A forms binding domains for carbohydrate structures that occur on the surface of the epithelium. Toxin B binds to cells that are not covered by a thick carbohydrate matrix and enter the cell by endocytosis. Both toxins require passage through an acidic intracellular compartment in order to intoxicate cells. Cells intoxicated by these proteins show retraction of cell processes and a rounding of the cell body. This is due to the disassembly of filamentous F-actin and an increase in G-actin prior to cell rounding. It has been proposed that *C. difficile* toxins act enzymatically within cells, modifying proteins that regulate actin polymerisation and fiber assembly. These proteins are known as the Rho proteins, a subfamily of the Ras-family of GTPases. Inactivation of the Rho, Rac or Cdc 42- family molecules which are present in the host epithelial cells leads to alterations in signaling and

disruption of barrier function which ultimately leads to apoptosis of the host cells (Fig. 2). [26, 30, 32]

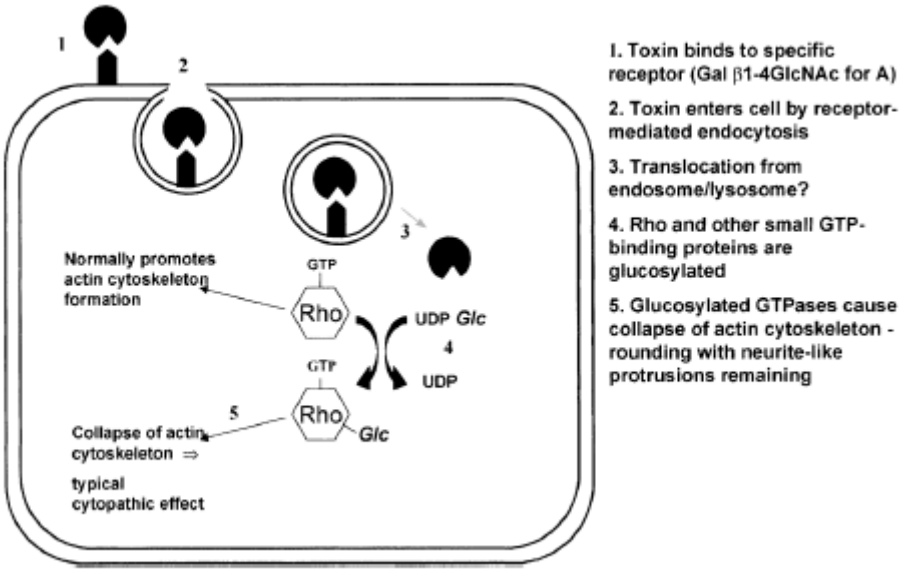


Figure 2: Action of *Clostridium difficile* toxins on the host epithelial cells (from ref. 30)

The barrier-disrupting effects of toxin A and B increase the colonic permeability, which is the basis of watery diarrhea, a typical feature of *C. difficile* antibiotic-associated diarrhea. In addition to the above mentioned cytotoxic effects, TcdA and TcdB also provoke inflammatory responses which eventually lead to tissue damage. Pseudomembranous colitis develops as a sequelae to this inflammation. Ng *et al.,* through studies on tissue culture models, demonstrated that toxins induce the production of the proinflammatory cytokine IL- 1β. Production of tumour necrosis factor α (TNF α) and IL-8 is also induced. This leads to tissue infiltration with neutrophils. Neutrophil recruitment appears to be an essential step in the pathogenesis of C. difficile toxin-induced intestinal injury as biopsy specimens from patients with C.

difficile colitis show marked vascular congestion, neutrophil infiltration of the lamina propria and inflammation. Once the inflammatory cascade is initiated, it can result in a marked acute inflammatory cell infiltration, further mucosal injury and focal pseudomembrane formation. For induction of the cytokines and activation of inflammation to take place, there needs to be intracellular recognition of the toxins followed by assembly of a multiprotein complex called the inflammasome. Studies on mouse models have shown that inhibition of this inflammasome assembly can prevent TcdA and TcdB mediated tissue damage. [26, 30]

All diarrheagenic strains of *C. difficile* produce Toxin B. In addition to this, some strains also produce Toxin A. In general, TcdB is considered to be more virulent than TcdA. Based on polymorphisms in the TcdA/ TcdB sequence, strains can be classified by ribotyping, pulsed field typing, restriction endonuclease analysis typing, multi locus repeat or non-repeat based sequence variations and surface protein variations typing. By these methods, newer epidemic strains have been identified as belonging to ribotypes 001, 017, 027, 078 and 106 (toxinotypes 0, VIII, III, V and 0, and pulse field types NAP2, NAP9, NAP1, NAP7/8 and NAP11 respectively). Ribotypes 001 and 078 are more prevalent worldwide but ribotype 027 is the most virulent strain. [26]

TcdC, TcdR, TcdE*:*

There are other genes that are located within the Pathogenicity Locus that are found to regulate the expression of TcdA and TcdB.

- TcdR- Facilitates the binding of RNA polymerase to the promoters of TcdA and TcdB genes. On reaching stationary growth, transcription of TcdA and TcdB is promoted by TcdR.
- TcdC- During exponential growth, higher levels of TcdC are expressed which act as an anti-sigma factor and suppress transcription of TcdA and TcdB. Many *C. difficile* isolates which are associated with epidemics have been found to possess a missense mutation in tcdC. Hypervirulent strains are associated with deletions in TcdC.
- TcdE- Encodes a holin- like protein that increases secretion of TcdA and TcdB.[26, 33]

Binary toxin:

Some strains produce another ADP- ribosylating toxin called the Binary toxin or *Clostridium difficile* transferase (CDT) that is not encoded in the pathogenicity locus. This has especially been noticed in association with the hypervirulent B1/NAP1/027 and 078 ribotype strains. CDT is composed of two proteins CdtA and CdtB. CdtA is an ADP ribosyl transferase that causes ADP ribosylation of actin leading to disruption and rearrangement of the host cell cytoskeleton. CdtB forms pores in the acidified endosome and facilitates entry of CdtA into the cytosol. Schwan *et al.* suggested that bacterial attachment to the host cells is enhanced by CDT. Ribosylation interferes with actin polymerization resulting in cellular protrusions caused by microtubules and enhanced fibronectin delivery to the cell surface, thereby increasing *C. difficile*

adhesion to the target cells. A study in Sweden concluded that CDT producing strains were associated with greater mortality in affected patients. [26, 33]

Non Toxin Virulence Factors:

Non toxin virulence factors have increasingly been found to play a role in the pathogenesis of CDI. They play a role in the colonization, proliferation and maintenance of *C. difficile* in the gut. [26]

Bile tolerance:

Studies on mice by Lewis *et al.* showed that isolates with greater tolerance to secondary bile acids like sodium deoxycholate were found to produce greater morbidity i.e. more prolonged and severe colitis. The germination and growth of *C. difficile* also depends on favourable concentrations of primary and secondary bile acids. The exact mechanism by which lithocholic acid tolerance in *C. difficile* increases in vivo disease severity is still poorly understood. [34]

Flagella:

One mechanism by which the intestinal cells are protected is by the production of mucus by the goblet cells. Tasteyre *et al.* were the first to identify the genes associated with flagella; the flagellin monomer (fliC) and the flagellar cap protein (fliD). Their study provided evidence that fliD plays a role in adhesion to mucus and both fliC and fliD are implicated in binding of *C. difficile* to the caecal tissue. [26]

Cell wall proteins:

Various structures and proteins on the surface of *C. difficile* contribute to pathogenesis. They include mechanisms which contribute to adhesion, tissue invasion, regulation of bacterial growth and metabolism and stimulation of the host immune response. One example is the fibronectin binding protein which is a 68kD magnesium containing protein that binds to fibronectin which is present on the host cell surface. This results in increased adherence to the host cells. [26]

C. difficile also has a surface layer (S- layer) that is composed of numerous proteins arranged in a crystalline lattice. Surface layer protein A (SlpA) is the predominant species. The S- layer has been implicated in adhesion, protein processing and cell wall regulation. These surface layer proteins (SLPs) belong to a larger group of molecules called cell wall proteins (CWPs). [26]

Host Resistance Mechanisms:

The various host resistance mechanisms are:

- Gastric acid in stomach
- Bile salts in small intestine
- Innate and adaptive immune response
- Microbiota of the colon

Gastric Acid in Stomach:

The vegetative forms of *C. difficile* are highly susceptible to gastric acid although the spores are resistant. Brown *et al.*, first described the association of acid suppressant medications with the development of CDI. [35, 36]

Bile Salts in Small Intestine:

The bile acid taurocholate acts as a germinant but chenodeoxycholate another bile acid decreases the affinity of *C. difficile* to taurocholate. In a normal, healthy host, the colonic microflora 7α-dehydroxylates chenodeoxycholate to lithocholate, which is also an inhibitor of germination. However, in an antibiotic-treated host, chenodeoxycholate is not 7α-dehydroxylated and has been shown to be absorbed by the colonic epithelium at a rate 10 times higher than that of cholate. Thus, in an antibiotic-treated individual, the ratio of cholate to chenodeoxycholate derivatives would favor germination. [37]

Innate and Adaptive Immune Response:

The host innate and adaptive immune response is not fully understood. The surface layer proteins have been found to induce the production of immunomodulatory cytokines such as IL- 1β, IL- 6 and IL- 10. As SLPs are immunodominant antigens, anti- SLP antibodies have been recovered from the serum of patient's with CDI. SLPs also have the ability to activate proinflammatory signaling, including those utilizing toll like receptor (TLR) signaling. TLR recognition of microbe associated molecular patterns results in

the engagement of adaptor molecules and subsequent signaling to activate inflammatory responses. Flagellin is also an activator of the innate immune response. Binding of flagellin to TLR5 on the host cells results in NFB activation and production of pro inflammatory cytokines. [26]

Microbiota of the Colon:

The mechanism by which the gut flora inhibits colonization by *C. difficile* is called colonization resistance. The various mechanisms by which the gut flora prevents colonization of *C. difficile* are:

- Inactivation of germinant molecules
- Occlusion of the host receptors needed for colonisation
- Production of toxic/ inhibitory substances which inhibit growth of *C. difficile*
- Stimulation of host responses to prevent establishment with *C. difficile*

All these above mentioned mechanisms act synergistically to prevent CDI. A limited number of bacteriocins have also been identified that exhibit antimicrobial activity against Gram-positive pathogens such as *C. difficile*. Chang *et al.* compared the gut microbiota of patients with initial CDI and recurrent CDI and compared it with healthy controls. Patients with recurrent CDI showed a marked decrease in flora especially in the phylum Bacteroidetes compared to healthy controls and even patients with initial CDI. To avoid recurrent CDI, it is essential to re-establish the gut flora. This is why fecal transplantation has become a successful option in the treatment of CDI. [26, 33]

Risk factors for the development of disease:

The risk factors for the development of CDI can be classified as primary and secondary risk factors. Primary risk factors include male gender, infants and elderly patients (>65 years), prolonged hospitalisation and antimicrobial therapy. Secondary risk factors include underlying comorbidities such as inflammatory bowel disease, prior gastrointestinal surgery, immunodeficiency, cystic fibrosis, diabetes mellitus, malnutrition, low serum albumin and neoplasia. Elderly patients with leukocytosis, hypoalbuminemia and on nasogastric feeding have higher mortality rates from CDI. [38] Administration of broad spectrum antibiotics that disturb the growth of normal flora is the most widely recognized risk factor. The antibiotics that have been implicated in CDI are cephalosporins, fluoroquinolones, clindamycin, ampicillin, amoxicillin, macrolides, cotrimoxazole and tetracycline. Even usage of metronidazole and vancomycin which are the drugs of choice for treatment of CDI may result in the disease. Apart from antibiotics, cancer chemotherapy drugs such as methotrexate, vinblastine, 5- fluorouracil, and cyclophosphamide have also been implicated in the development of CDI. There have been reports that usage of proton pump inhibitors and H2 blockers may also lead to disease. Some studies have shown that consumption of contaminated meat and other food may serve as a risk factor in causing community acquired CDI (CA- CDI). [1, 39]

DISEASES CAUSED BY *Clostridium difficile:*

Clostridium difficile is most commonly implicated as the cause of infectious diarrhea in hospitalized patients. Hospital acquired CDI (HA- CDI) is defined as onset of symptoms in patients 48 hours following admission to a health care facility and less than 4 weeks following discharge. [(1)] Infection with this organism presents with a wide spectrum of manifestations clinically ranging from asymptomatic colonization to mild diarrhea to life threatening illness.

Carrier stage: Individuals who shed *C. difficile* in their stool without diarrhea are considered to be the reservoirs of infection. Various studies have shown the carriage rate to be 3%, 20-30% and 50% in healthy adults, hospitalized patients and following prolonged hospitalization respectively. 10-16% of patients in high risk units become carriers following administration of antibiotics. Carrier stage occurs due to the development of a serum IgG response to the enterotoxin in patients with *C. difficile* colonization. [(7)] Carriers facilitate the spread of the spores into the environment at a rate lower than patients with clinical manifestations. [(1)]

***C. difficile* associated diarrhea (CDAD):** This accounts for almost 25-30% of cases in patients with antibiotic associated diarrhea. It can occur within 2 hours of initiating antibiotics. Diarrhea is defined as passage of unformed stools more than thrice a day for atleast two consecutive days. Patients present with uncomplicated watery diarrhea, abdominal pain and cramps. In such patients,

Toxin A is commonly identified in the stool but endoscopic and histological findings may be normal. [1, 7, 40]

***C. difficile* associated colitis (CDAC):** Progression to massive colonic inflammation following diarrhea is defined as colitis. Pseudomembrane formation is not seen in this group of patients. Patients present with abdominal pain, nausea, malaise, anorexia, watery diarrhea, traces of blood in stool, low grade fever, dehydration and leucocytosis. [1, 40]

Pseudomembranous colitis (PMC): Pseudomembranous colitis is the advanced stage of the disease and is diagnostic of CDI. Lesions begin to form in the intestine which coalesce together to create a pseudomembrane that is made up of immune cells, mucous and necrotic tissue. Sigmoidoscopy shows 2-10mm yellowish plaques in the colorectal mucosa and occasionally in the terminal ileum. [1, 40]

Fulminant colitis: 3% of patients develop severe or fulminant colitis and present with signs of systemic toxicity or systemic inflammatory response syndrome (SIRS). These patients have leukocytosis, elevated serum lactate levels, acute renal failure, hypotension and respiratory distress. Such patients require colectomy. However, despite surgery and antibiotic therapy, the mortality rate in such patients is as high as 67%. Patients develop multiorgan failure and perforation of the intestine leading to death. [7] Some patients develop toxic megacolon which is characterized by a distended colon. 20% of patients with advanced disease do not have diarrhea and instead present with

abdominal distention and ileus, leading to a misdiagnosis. The factors that lead to a rapid transition from mild disease to fulminant colitis is unfortunately not known. An increased rate of fulminant colitis has been noted in recent years since the emergence of hypervirulent strains [1, 40]

Recurrent CDI: This occurs due to relapse or reinfection with a different strain. Various studies have shown the rate of recurrence to be between 33-75%. Twenty five percent of patients develop recurrence 4 weeks after completing treatment with metronidazole or vancomycin. The reason for relapse is poorly understood but maybe to alteration in the normal gut flora or a defective immune response against the bacteria and its toxins. The small bowel and appendix may also act as a reservoir for the spores which enter the colon and cause relapse. [1, 7]

Extracolonic manifestations: Extracolonic manifestation that have been reported include formation of pseudomembranes on the ileal mucosa, bacteremia, reactive arthritis, visceral and intraabdominal abscesses, osteomyelitis, empyema and appendicitis. Patients who develop extracolonic manifestations usually have underlying gastrointestinal disease, anatomical disruption or have undergone surgery of the colon. [1]

DIAGNOSIS:

The aim of diagnostic tests is to differentiate between colonization and infection with *Clostridium difficile*. The various tests that are available are:

1. **Cell culture cytotoxicity neutralization assay (CCCNA):** In the past, this was considered as the gold standard for the diagnosis of CDI. In this test, stool filtrates are inoculated over a monolayer of cells with and without *C. difficile* antitoxin. A number of different cell lines have been used such as human foreskin fibroblasts, human diploid fibroblasts, MRC-5 lung fibroblasts, Vero cells, Hep2 cells and McCoy cells. Rounding of cells in the wells without antitoxin demonstrates the cytopathic effect of the toxin. Absence of cytopathic effect in the wells with antitoxin confirms the presence of *C. difficile* toxin in the stool. This test is quite specific and can detect toxin levels as low as 10 picogram. However, sensitivity ranges from 65-90%. Degradation of the toxin can occur if there is a delay in the transportation of the specimen, leading to false negative results. The results also vary depending on the type of cell line used. Due to the lack of standardization, cost and slow turnaround time it has been replaced by other diagnostic tests. [12, 41]
2. **Toxigenic culture (TC):** The Infectious Diseases Society of America (IDSA) 2010 guidelines consider this as the gold standard against which all tests should be compared. In this method, stools samples are cultured onto the selective medium CCFA (cycloserine cefoxitin fructose agar) and if growth occurs, further testing for toxin production is done. Perirectal or rectal swabs can also be used as the specimen. In comparison to TC, CCCNA has a lower sensitivity of 67-79%. But TC only detects the ability of the organism to produce toxin and not the

actual presence of toxin in stool. Therefore, 7-20% of false positives are seen with this method as the patient might be asymptomatic and just colonized with a toxigenic strain of *C. difficile*. Currently, it is undecided as to whether TC or CCCNA would be a better gold standard, with many reports stating that a gold standard does not exist. [12, 41]

3. **Enzyme immunoassays (EIA):** EIAs for Toxin A/B currently is the primary test that is used in most laboratories as it is rapid, inexpensive and convenient to perform. These tests use monoclonal or polyclonal antibodies that are targeted against the toxin. But it is insensitive and non- specific. It requires 100- 1000 picogram of toxin compared to CCCNA which can detect much lower levels. This has led to the two step testing strategy where detection of glutamate dehydrogenase (GDH) is first done. GDH is a metabolic enzyme encoded by gluD. This antigen is expressed in high levels by all strains of *C. difficile.* However, detection of GDH antigen only confirms the presence of *C. difficile* in stool and does not confirm toxin production. Therefore, in the second step, testing for toxin production is done. With this testing strategy, sensitivities as high as 100% have been reported. [12, 41]
4. **Polymerase chain reaction (PCR):** Use of PCR for detection of tcdB which encodes Toxin B is promising as a standalone test. It is fast, sensitive but more expensive than EIAs (Table 2). Sensitivity is reported to be 84- 94% in comparison to TC. Currently, there are 4 Food and Drug administration (FDA) approved PCR assays- Gene Ohm, Gene

Xpert, Progastro and Simplexa. But like TC, it only detects the toxin gene leading to more false positives. It is also not recommended in diagnosis of suspected relapse, as 56% of patients will be positive by PCR 1-4 weeks after therapy. ACG 2013 guidelines recommend using nucleic acid amplification techniques (NAAT) as part of a three step testing strategy. The first step is testing for GDH antigen, followed by EIA for toxin A/B. If the second step is negative, absence of toxin should be confirmed by NAAT in the third step. [41, 42]

Table 2: Comparison of the various laboratory tests for diagnosis of CDI [40, 42]

	Sensitivity	**Turnaround time**	**Cost**	**Availability**
EIA Toxin A/B	++	Hours	+	++++
GDH	++++	Hours	+	++++
PCR	+++	Hours	++++	+++
TC	+++++	Days	+++	+
CCCNA	+++	Days	+++	+

5. **Loop mediated isothermal amplification (LAMP):** It is a non PCR based gene amplification method that detects the pathogenicity locus of toxigenic *C. difficile.* It is simple, rapid and less expensive than PCR. However, like PCR it only detects the toxin genes. The Illumigene assay was found to have a sensitivity of 92%, specificity of 98%, 99% negative predictive value and 84% positive predictive value. [41]

6. **Radiologic studies:** These are of little value and have non-specific findings. Plain films of the abdomen reveal colonic dilatation and non-obstructive small bowel airfluid levels. In 39% of cases, abdominal computed tomography (CT) scans may be normal. It may show localized or diffuse thickening of the colonic wall. With fulminant colitis, mucosal “thumbprinting” and “accordion” sign is seen where there is trapping of the oral contrast within the thickened mucosal folds. (41)

7. **Endoscopy:** American College of Gastroenterology (ACG) recommends endoscopy only when a rapid diagnosis is needed and there is a delay in the results of toxin assay or there is a negative toxin assay but high suspicion of CDI still remains. It is also recommended in patients with ileus who are unable to pass stool. Frequently, the endoscopy is normal in patients with mild disease. In advanced disease, pseudomembranes are seen as yellowish white plaques which are elevated above the surrounding mucosa. Their measurement varies from a few millimeters to 20 mm. As the disease progresses, the plaques become confluent and they slough off leaving behind a denuded mucosa. Intervening mucosa may be normal, erythematous or edematous. Histopathological examination may also be done which shows pseudomembranes composed of fibrin, mucus, epithelial cells and inflammatory cells. (41)

EPIDEMIOLOGY:

C. difficile is a major cause of antibiotic associated diarrhea and accounts for about 15-25% of cases worldwide. [43] A few studies in the United States even suggest that *C. difficile* has overtaken methicillin resistant *Staphylococcus aureus* (MRSA) as the most common hospital acquired infection. [44] Between 1991 and 2003, it was discovered that the rate of CDI had almost doubled from 65.6 to 156.3 per 100,000 population. An increase in the number of patients with severe CDI and need for colectomy was also noted in Quebec, Canada in 2002. Additionally, the proportion of patients who had complicated cases (defined as development of megacolon, perforation, colectomy, shock requiring vasopressor support, mortality) increased from 7.1 to 18.2 percent ($P<0.001$) and 30-day mortality increased from 4.7 to 13.8 percent ($P<0.001$). This rise in the number of cases of CDI was later attributed to the emergence of the hypervirulent strain, designated restriction endonuclease analysis type BI, North American pulsed-field gel electrophoresis type 1 (NAP1), polymerase chain reaction (PCR) ribotype 027 (i.e. BI/NAP1/027). [45]

In comparison to the United States, where most hospitals have guidelines for the diagnosis and prevention of CDI, a lack of awareness has been observed among healthcare professionals in Asia. Hence, limited studies are available in Asia, including India. This may also be due to the lack of technology and facilties that are available for culturing anaerobic organisms. Prevalence of CDAD in India is similar to other studies worldwide. *C. difficile* was isolated from 25.3% patients (of all age groups) with diarrhoea in a study by Gupta and

Yadhav. In 7.3% of acute diarrhea patients, *C. difficile* was identified as the sole pathogen in a study by Dutta *et al.*, of which 82.4% were cytotoxin producers. In one study, Niyogi and Bhattacharya *et al.* isolated *C. difficile* from fecal samples from 11% hospitalized patients with diarrhoea and 2.9% from patients without diarrhea. 87% of these isolates were reported to produce cytotoxin; however the patients with diarrhoea had no history of antibiotic usage. Kochhar *et al.* verified that *C. difficile* was responsible for some of the exacerbations in ulcerative colitis patients even though there was no history of recent antimicrobial exposure or hospitalization. Vaishnavi *et al.* in 2011 reported that *C. difficile* toxin test was positive for 30% of hospitalized patients receiving single to multiple antibiotics for different diseases (of all age group), but only for 7% patients not receiving antibiotics. [4, 7]

More recently, the number of CDI-related hospital stays appears to have leveled off between 2008 and 2010. Similar trends have been noted in parts of Europe since 2007. From 2007 to 2010, a 61 percent reduction in the incidence of CDI was observed in England. In India, Chaudhry *et al.* reported that number of *C. difficile* positive cases have decreased during a 5 year study period. The decreased incidence is stipulated to be due to the success of expanded prevention and control efforts, improved antibiotic policy, changes in the prevalence of epidemic strains (i.e., ribotype 027) or perhaps a combination of factors. [4, 43]

C. difficile infection disproportionately affects older patients with dramatic differences observed in those ≥65 years of age. In the U.S., a total of

93 percent of deaths from CDI occurred in patients ≥65 years of age and was reported as the 18th leading cause of death in this age group in 2008. Although, overall a decrease in the incidence of CDI has been reported, an apparent increase in the incidence among populations in the community who were historically considered to be at low risk, such as healthy peripartum women, children, antibiotic-naïve patients, and those with minimal or no recent healthcare exposure has been reported. (43)

Recent studies also suggest that CDI is not merely acquired from hospitals but can also be acquired in the community. This might be due to antibiotic exposure and usage of proton pump inhibitors in outpatients too. (44) Data from North America and Europe suggest that approximately 20–27 percent of all CDI cases are community-associated, with an incidence of 20–30 per 100,000 population. Centers for Disease Control and Prevention (CDC) launched an active, population-based surveillance to identify cases of CA-CDI. Community-associated CDI cases were defined if a positive stool specimen was collected as an outpatient or within 3 days of an acute care admission in a patient without documentation of an overnight stay in a healthcare-facility in the prior 12 weeks. The ribotype 027 strain was the most prevalent strain identified in the CA-CDI population. (43)

C. difficile infection places a significant economic burden on the healthcare system. The acute-care direct costs of CDI in the U.S. were estimated to be $4.8 billion in 2008. The average duration of hospital stays during which CDI was a secondary diagnosis were more than twice as long as

those with CDI as a principle diagnosis (16.0 vs. 6.9 days) and costs were more than three times higher ($31,500 vs. $10,100). However, the actual cost is likely to be higher and more data is needed to assess healthcare cost. Furthermore, nearly all the published studies have focused on CDI diagnosed and treated in acute-care hospitals and fail to measure the burden outside the hospital, including recently discharged patients, outpatients, and those in long-term care facilities. [43]

TREATMENT:

The initial measure that needs to be taken in a patient with CDI is to stop the precipitating antibiotic and start the patient on intravenous fluids and electrolytes. Antiperistaltic agents are not recommended as they can predispose the patient to the development of toxic megacolon. Antibiotics are the mainstay of treatment of CDI. [46]

Oral vancomycin was the first drug shown to be effective for the treatment of CDI. Later metronidazole was found to be equally effective and is now the drug of choice in the treatment of CDI. The reason why metronidazole is preferred is because it is cheaper and due to concerns that vancomycin treatment may lead to the emergence of vancomycin resistant enterococci. Metronidazole is however contraindicated in pregnant and lactating women. [46]

Recently revised treatment guidelines for CDI classify patients into three groups:

- Mild to moderate disease- Oral metronidazole at a dose of 500 mg 3 times per day is recommended as first-line therapy
- Severe disease (white blood cell count >15,000 cells/mm3 or creatinine level >1.5 times the level prior to CDI)- Oral vancomycin 125 mg 4 times daily for 10–14 days is recommended
- Severe complicated disease (severe disease and admission to an intensive care unit, need for colectomy, toxic megacolon, ileus, hypotension or, colonic perforation)- Vancomycin 500 mg orally or via nasogastric tube 4 times per day and/or intravenous metronidazole 500–750 mg every 8 hours is recommended.
- If a patient with severe complicated CDI has complete ileus, intravenous metronidazole is used with rectal administration of vancomycin. [46]

CDC guidelines also recommend that in case of recurrence, the patient should be treated with the same drug that was used initially for treatment. For a second recurrence, metronidazole is not recommended. Oral vancomycin with or without pulse dosing is preferred in such cases. Peláez *et al.* conducted *C. difficile* susceptibility tests over an eight-year period and reported 6.3% of isolates as resistant to metronidazole (minimum inhibitory concentration (MIC) > 32 μg/mL), 3.1% of isolates intermediately resistant (MIC 4–16 μg /ml) to vancomycin and 0 isolates resistant to vancomycin (MIC > 32 μg/mL). [46]

Alternative therapeutic options for the treatment of CDI include other antimicrobial agents, toxin binding drugs, probiotics and immune modifying agents. Most of the antimicrobial agents are still being evaluated for their

efficacy in the treatment of CDI. These include Ramoplanin, Rifaximin, Nitazoxanide, Fusidic acid, Teicoplanin and Rifampin. Rifaximin was found to be useful in treating a refractory case of CDI. [46]

A novel macrocyclic agent that has been developed for the treatment of CDI is Fidaxomycin. This drug has a narrow spectrum of activity. It has also been shown to reduce the risk of recurrent infection and also eliminate *C. difficile* spores. The dosage is 200mg BD for 10 days in recurrent infections. However, the cost is seven times that of Vancomycin, thereby limiting its usage. [47]

The toxin binding or anion binding resins are Cholestyramine, Colestipol and Tolevamer. They bind to toxins A and B, thereby reducing fluid accumulation due to Toxin A. Several case reports are available that mention the efficacy of these agents in treating relapse in patients who fail to respond to traditional therapy. [46]

Research has begun on the use of immune modulating agents in the treatment of CDI. Two human monoclonal antibodies, CDA1 and MDX-1388, which are directed against *C. difficile* toxins A or B respectively are being studied in clinical trials. [46]

High levels of anti-toxin A IgG are associated with protection against CDI. An anti-*C. difficile* toxoid has been developed to determine if it is possible to induce an immune response in patients with multiple episodes of

recurrent CDI. This vaccine consists of formalin detoxified *C. difficile* toxins A and B which is believed to induce anti- *C.difficile* toxin IgG. [46]

Probiotics serve as a potential method of restoring normal flora in the gastrointestinal tract, which is lost while treating patients with antibiotics. Bacterial strains of *Lactobacillus* and *Bifidobacterium* and the yeast *Saccharomyces* are commonly utilized in the prevention and treatment of CDI. Studies have shown probiotics to be relatively safe. Bovine colostrum and Whey protein concentrate are also useful as an adjunctive measure in the treatment of CDI. [46]

Aas *et al.*, in 2003, first described the efficacy of fecal microbiota transplantation (FMT) in the treatment of recurrent CDI. The fecal transplant is administered via a nasogastric tube, enema or colonoscopy. It can be mixed with saline, yoghurt, milk or water to reduce the odour. This technique restores the bacterial flora and prevents overgrowth of *C. difficile.* To minimize risk of transmission of other diseases, stool donation is preferred from a close relative of the patient. Recently, fecal material contained in capsules has been developed. This is a more aesthetically acceptable route of FMT. [46, 47]

Surgery is only recommended in severe, complicated cases of CDI. Total colectomy is preferred over partial colon resection as post-surgical mortality rates are quite high with the latter procedure. [46]

PREVENTION AND CONTROL:

The major source of transmission of infection is from patients with symptomatic CDI. These patients shed the spores in large numbers in their feces resulting in contamination of their skin, clothes, bedding and surrounding environment. Healthcare workers then serve as a vector and transmit the spores through their contaminated hands to susceptible patients. So the mainstay of prevention of CDI is to reduce transmission of the spores. IDSA recommends that all patients with CDI be placed under contact precautions until the diarrhea resolves. A separation of minimum 3 feet between beds is recommended. Following discharge of the patient, disinfection of the room including bedding and portable equipment must be done using a sporicidal agent such as sodium hypochlorite. [48, 49]

Primary prevention is ensuring that the patient's normal gut flora is not disrupted and that they do not become susceptible to CDI. To realize this goal, it is crucial that every hospital formulates its own antibiotic policy which includes measures to control unnecessary usage of antibiotics. Whenever possible, narrow spectrum antibiotics must be used. Reduced usage of antibiotics that are commonly implicated in the development of CDI is recommended. Regular prescription review must be done to ensure that antibiotics are discontinued as soon as possible. [50, 51]

The other aspect of primary prevention and infection control is handwashing. Some reports suggest that the spores are resistant to alcohol and

most of the commonly used disinfectants. Ordinary soap and water as well as 4% chlorhexidine are equally effective in removing the spores of *C. difficile* from the hands. It is essential that all healthcare workers wash their hands after touching a patient and they should be educated regarding the same from time to time. [50]

MATERIALS AND METHODS

This prospective observational study was conducted in the Department of Microbiology, PSGIMS&R after obtaining the Institutional Human Ethical Committee's approval.

STUDY PERIOD: January 2016- August 2017

SAMPLE SIZE:

Consecutive stool samples were collected from 135 hospitalized patients with antibiotic associated diarrhea, who were clinically suspected to have *Clostridium difficile* infection at PSG Hospitals, Coimbatore.

SAMPLE SIZE JUSTIFICATION:

Formula used: $n= t^2 \times p\ (1-p)/m^2$

Where;

n= required sample size

t= confidence level at 95% (standard value of 1.96)

p= estimated prevalence of *C. difficile*

m= margin of error at 5% (standard value of 0.05)

Estimated prevalence from our hospital statistics (p) = 0.1

n= 1.96 x 1.96 x 0.1 (1- 0.1)/ 0.05 x 0.05

n= 138 (135)

INCLUSION CRITERIA:

Hospitalised patients on antibiotics, who developed diarrhea and were clinically suspected to have *Clostridium difficile* infection were included in the study. Diarrhea was defined as passage of loose or watery stools, or frequency of stools > 3 times per day.

EXCLUSION CRITERIA:

- Patients who had been hospitalized for < 3 days
- Patients not on antibiotics
- Infants (< 1 year of age)

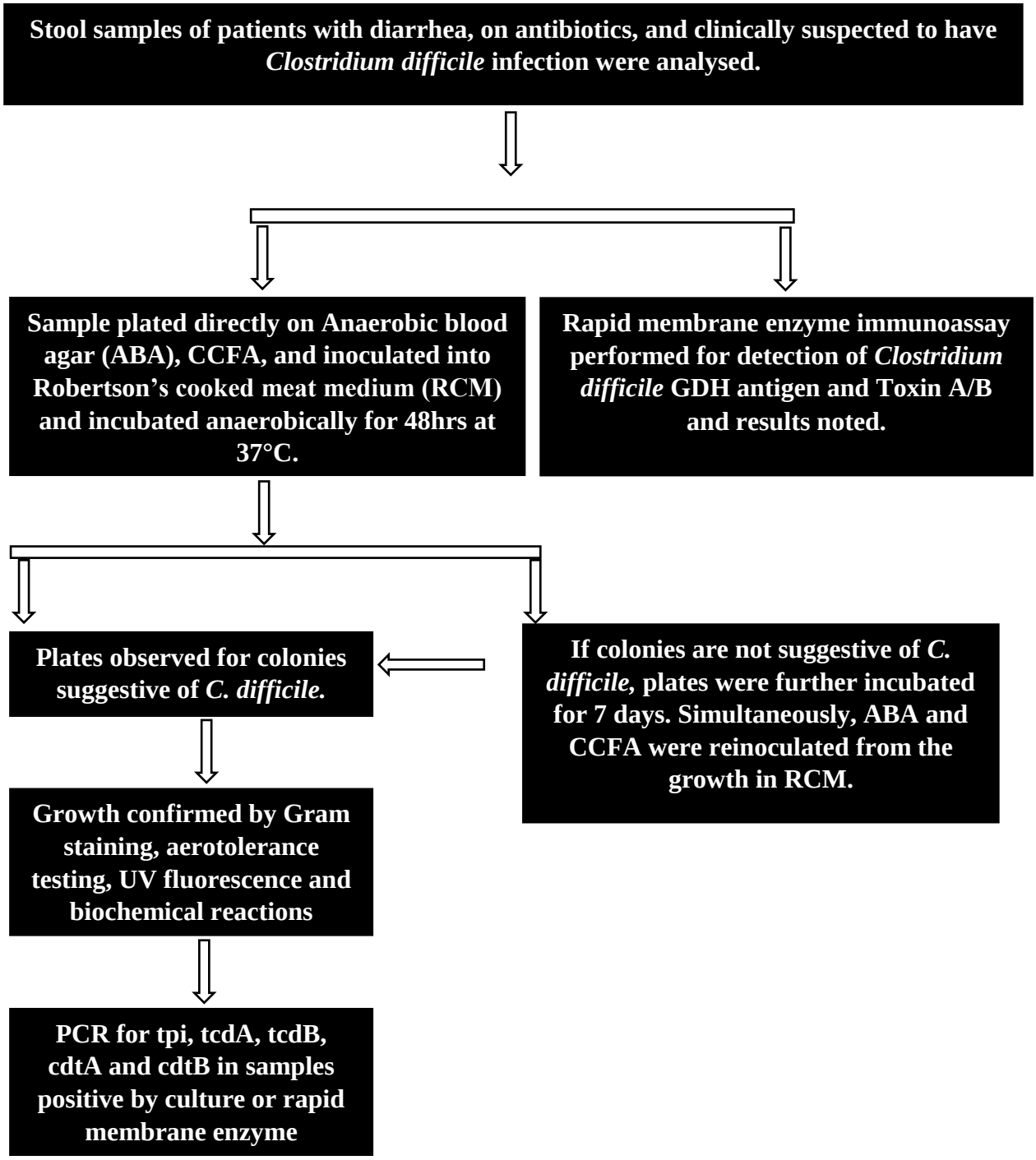
ETHICAL CLEARANCE:

This prospective study was approved by the Ethical Committee of PSG IMS&R.

STATISTICAL ANALYSIS:

Statistical analysis was performed using IBM SPSS software (Statistical Product and Services Solutions, version 24, SPSS Inc, Chicago, Il, USA) to analyse data. Comparison between enzyme immunoassay and culture for detection of *C. difficile* was tested independently using Pearson Chi^{-} squared test. Results with p- values less than 0.05 were considered statistically significant.

Figure 3: WORK FLOW DIAGRAM



CULTURE:

Stool samples from patients who were clinically suspected to have *C. difficile* infection were transported to the laboratory in a universal container. Demographic information including antibiotic usage and signs and symptoms of CDI in the patient were recorded. Faecal specimens were cultured within 24 hours of receipt. [52] In case there was a delay in processing, the samples were stored at 2- 8°C for a maximum of 3 days. Using a 10µl inoculating loop, 1 loopful of unformed stools was plated onto the non-selective medium Anaerobic blood agar, the selective medium Cycloserine-Cefoxitin Fructose agar, and inoculated in a broth of Robertson's cooked meat medium for enrichment and as backup in case of jar failure. The culture plates were incubated at 37°C for 48 hours under anaerobic conditions i.e. 0.2% O_2, 9.9% H_2, 9.9% CO_2 and 80% N_2, using the Anoxomat Mart II system (Mart Microbiology B.V., Netherlands). Simmon's citrate medium which was inoculated with *Pseudomonas aeruginosa* was used as a bacteriological indicator to check for strict anaerobiosis. Change in the colour of the medium to blue, indicates failure to achieve anaerobiosis. In such a situation, plating was repeated. After 48 hours, the plates were checked for growth. In case, growth was not suggestive of *C. difficile*, the plates were further incubated. Simultaneously, the sample was plated again onto Anaerobic blood agar and CCFA from the growth in RCM. All plates without growth suggestive of *C. difficile* were discarded after 7 days of incubation.

Identification was confirmed by the cultural characteristics on Anaerobic blood agar, CCFA, fluorescence under UV light, Gram stain appearance, aerotolerance testing, metronidazole sensitivity and biochemical reactions.

Aerotolerance testing was done for all suspected colonies by subculturing the colony onto two blood agar plates. One of the plates was incubated aerobically and the other anaerobically. Metronidazole (5μg) disc was put in the beginning of the second streak line on the plate that was incubated anaerobically to presumptively identify anaerobic growth. Failure to grow aerobically helped to rule out facultative anaerobes.

To perform Gram stain, a single colony was emulsified in a drop of saline on a clean, glass slide. The smear was air dried, heat fixed and then stained. On Gram staining, *C. difficile* appears as large, Gram positive rods with subterminal spores seen occasionally.

C. difficile is catalase and oxidase negative. Catalase test was performed by picking a colony with the help of a clean capillary tube and immersing it in a test tube containing 3% hydrogen peroxide. Lack of production of effervescence within 20 to 30 seconds indicates a negative reaction. Oxidase test was performed by smearing a colony onto a dry filter paper impregnated with tetramethyl paraphenylene diamine dihydrochloride. No change in colour from white to purple within 10 seconds indicates a negative reaction. [53]

Fermentation of carbohydrates was tested by inoculating a heavy suspension of the suspected colonies into thioglycollate broth containing 1% of

the carbohydrate. This was incubated anaerobically at 37°C for 48hrs. Positive results are indicated by a change in the colour of the solution to yellow on addition of Bromothymol blue indicator. Negative results are indicated by the change in colour to green. The carbohydrates that were tested were Glucose, Lactose, Sucrose, Maltose, Mannitol, Fructose and Xylose.

Esculin hydrolysis was tested by inoculation the suspected colony into esculin broth and incubating this solution anaerobically at 37°C for 48hrs. Change in the colour of the solution to black on addition of ferric ammonium citrate indicates a positive result.

To test for lipase and lecithinase activity, the colony was inoculated on to egg yolk medium and incubated anaerobically for 48hrs at 37°C. Lipase reaction is considered to be positive if oil on water appearance occurs. Lecithinase reaction is considered to be positive if a white opacity surrounds the inoculum.

Once the growth was confirmed, the isolates were stored in glycerol stock solution. This was done by picking an individual colony and suspending it in Brain heart infusion (BHI) broth and incubating it anaerobically till the solution turned turbid. This was then added to glycerol such that a 15% glycerol stock solution was prepared. Long term storage of the isolate was done by storing this solution at -80°C. Recovery of the isolate was done by thawing the frozen stock and inoculating a loopful of the solution in BHI broth

supplemented with sodium taurocholate. Once turbid, it was plated on the culture media.[54]

RAPID MEMBRANE ENZYME IMMUNOASSAY (EIA):

Rapid membrane enzyme immunoassay was performed using Techlab C. diff Quik Chek Complete (Alere, Florida, USA).

Materials provided in the kit:

1. Membrane device
2. Diluent
3. Wash buffer
4. Substrate
5. Enzyme conjugate
6. Positive control
7. Graduated disposable transfer pipettes

Materials required:

1. Fecal specimen
2. Test tube
3. Disposable gloves

All the necessary reagents that were provided in this kit were brought to room temperature prior to the test. In a small test tube, 750µl of the diluent is added. To this, 1 drop of the conjugate is added. Using the graduated pipette, 25µl of freshly collected fecal specimen is transferred to the diluent/ conjugate mixture. 500µl of the diluted sample- conjugate mixture is transferred to the sample well on the membrane device. The device is incubated at room temperature for 15 minutes. 300µl of the wash buffer is then added to the reaction window on the device. Following complete absorption of the buffer, 2

drops of the substrate is added to the reaction window. Results are read and interpreted after 10 minutes.

The reaction window on the device has three vertical lines of immobilized antibodies. The antigen test line ("Ag") contains antibodies against *C. difficile* glutamate dehydrogenase. The control line ("C") is a dotted line that contains anti-horseradish peroxidase (HRP) antibodies. The toxins A and B test line ("Tox") contains antibodies against *C. difficile* toxins A and B. The conjugate consists of antibodies to glutamate dehydrogenase and antibodies to toxins A and B coupled to horseradish peroxidase. When the diluted sample- conjugate mixture is added to the well, any glutamate dehydrogenase and toxins A and B in the sample bind to the antibody-peroxidase conjugates. The antigen-antibody-conjugate complexes migrate through a filter pad to a membrane where they are captured by the immobilized glutamate dehydrogenase-specific and toxins A and B-specific antibodies in the lines. [55]

Interpretation:

- Negative result- Single blue dotted line in the middle of the reaction window below "C" on the device
- Positive antigen result- Blue line below "Ag" on the device along with the dotted blue line below "C"
- Positive antigen and toxin result- Two blue lines below "Ag" and "Tox" on the device along with the dotted blue line below "C"

- Invalid result- Absence of the blue dotted line below “C”

Before opening each lot, the device and reagents were validated by testing with the positive control that was provided and using the diluent as a negative control. Only if the controls were satisfactory, patient’s samples were tested.

POLYMERASE CHAIN REACTION (PCR):

PCR was done for all the isolates which grew on culture to check for the presence of toxigenic genes. Extraction of DNA was done prior to PCR using Quick- gDNA Miniprep Kit (Zymo Research, USA). The kit can be stored at room temperature (15-25°C).

DNA extraction:

Materials provided in the kit

- Genomic lysis buffer
- Prewash buffer
- Wash buffer
- Elution buffer
- Spin column
- Collection tubes

Other materials required:

-Vortex mixer

- Microcentrifuge
- 0.5 ml Eppendorf tubes
- Micropipettes (1000µl, 100µl)
- Microtips (1000µl, 100µl)

Procedure:

1. A loopful of *C. difficile* colonies is suspended in 100µL of saline. 400µL of the Genomic lysis buffer (4:1 ratio) is added to this.

2. The solution is vortexed for 4-6 seconds and kept at room temperature for 10 minutes.
3. This mixture is then transferred to the spin column in a collection tube and centrifuged at 10,000rpm for 1 minute.
4. The collection tube with flow through is discarded and the spin column is transferred to a new collection tube.
5. 200µl of Prewash buffer is added and the mixture is centrifuged at 10,000rpm for 1 minute. 500µl of Wash buffer is added to the spin column and centrifuged again for 1 minute.
6. The spin column is transferred to a new collection tube and 50µL of the Elution buffer is added. This is incubated for 5 minutes at room temperature. Centrifugation was done at 12,500rpm for 1 minute to elute the DNA.
7. The eluted DNA was stored in 0.5ml Eppendorf tubes at -20°C.
8. Direct extraction of DNA from the stool specimen was performed in EIA positive as well as culture positive samples. In this method, 1 loopful of the stool sample was suspended in 100µl of saline in an Eppendorf tube and 400µl of the Genomic lysis buffer was added to it. The solution was vortexed for 4-6 seconds and allowed to stand at room temperature for 10 minutes. Centrifugation of the lysate was done at 10,000rpm for 5 minutes. Without disturbing the pelleted debris, the supernatant was then transferred to a spin column and steps 3-7 were performed.

PCR:

PCR was done for positive isolates for the detection of tpi gene which is a housekeeping gene for *C. difficile* and for the toxigenic genes tcdA, tcdB, cdtA and cdtB. Based on previous studies, the primer sequence was obtained for these genes and acquired from Sigma- Aldrich, Bangalore (Table 3).

Table 3: Primer sequence

Gene	**Primer sequence (5'-3')**	**Amplicon size (bp)**	**Reference**
tpi	F- AAAGAAGCTACTAAGGGTACAAA R- CATAATATTGGGTCTATTCCTAC	230	56
tcdA	F- ATGATAAGGCAACTTCAGTGG R- TAAGTTCCTCCTGCTCCATCAA	624	57
tcdB	F- AAGGTTTATATGGATGAT R- TACAACTTTATTAACACAACT	591	58
cdtA	F- TGAACCTGGAAAAGGTGATG R- AGGATTATTTACTGGACCATTTG	353	59
cdtB	F- CTTAATGCAAGTAAATACTGAG R- AACGGATCTCTTGCTTCAGTC	490	59

The deprotected and desalted primers were resuspended in 1xTE (10mM Tris, pH 7.5-8.0, 1mM EDTA) and stored at -20°C.

Reaction mixture:

Each single reaction mixture (25µl) contained 3µl of DNA suspension, 12.5µl of Master Mix (EmeraldAmp GT PCR Master Mix composed of DNA polymerase, dNTPs, optimized reaction buffer, density reagent and green dye), 0.5µl of the forward primer and 0.5µl of the reverse primer. The primers were diluted 1:10 with molecular grade water before use. The remaining volume was adjusted with PCR grade water.

PCR:

The PCR was performed by conventional method using Applied Biosystems Step One Realtime PCR system under the following conditions:

For the identification of tpi, tcdA and tcdB genes; initial denaturation was done at 94°for 5 minutes, 35 cycles of 94°C for 30 seconds, annealing temperature 52°C for 45 seconds, extension at 72°C for 45 seconds and a final elongation at 72°C for 5 minutes.

For cdtA and cdtB genes, amplification was performed for 35 cycles of 94°C for 45 seconds, the annealing temperature was changed to 55°C for 30 seconds and extension at 72°C for 30 seconds.

The amplified products were stored at -20°C until they were subjected to gel electrophoresis.

Agarose gel electrophoresis:

The amplified products were visualized by agarose gel electrophoresis. 1.8% agarose gel was prepared by adding nuclease and protease free agarose powder with Tris Borate EDTA (TBE) buffer. This mixture was heated in the microwave for 3 minutes and 0.5µl of ethidium bromide was added to visualise the amplified DNA under UV light. The mixture was allowed to set in an electrophoresis tank with a comb in place.

A 100 base pair ladder was used as the molecular marker to measure the size of the amplified product and was added in the first well. The rest of the wells had 4µl of the amplified product. Gel electrophoresis was performed by placing the gel in an electrophoresis tank containing TBE buffer at 100 volts for 1 hour.

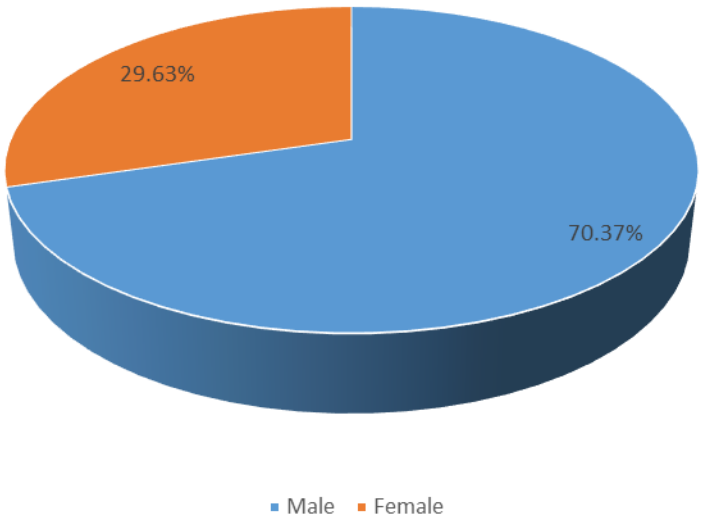
Interpretation:

Following electrophoresis, images of the gel were captured by Gel Doc. By comparing with the 100 base pair ladder, the size of the amplified product was measured and noted down.

RESULTS

Consecutive samples from patients with antibiotic associated diarrhea and who were suspected to have *C. difficile* infection were processed. Among these, 135 samples met the inclusion criteria. Of these, majority of the samples were obtained from male patients (70.37%), with 29.63% of the samples being obtained from female patients (29.63%). Figure 4 shows the increased incidence of nosocomial diarrhea among male patients compared to female patients.

Figure 4: Sexwise distribution of study group



The patients were analysed based on their age. The mean age of the patients was 52.55 years with a standard deviation (S.D.) of 16.19 years. Maximum number of patients were under the age group of 56-65 years i.e. 37

(27.41%) patients, followed by 27 (20%) patients between 46-55 years. The study population belonging to the extremes of age i.e. between 16-25 years and 76- 85 years showed the least incidence of 5.19% and 6.67% respectively as shown in Figure 5.

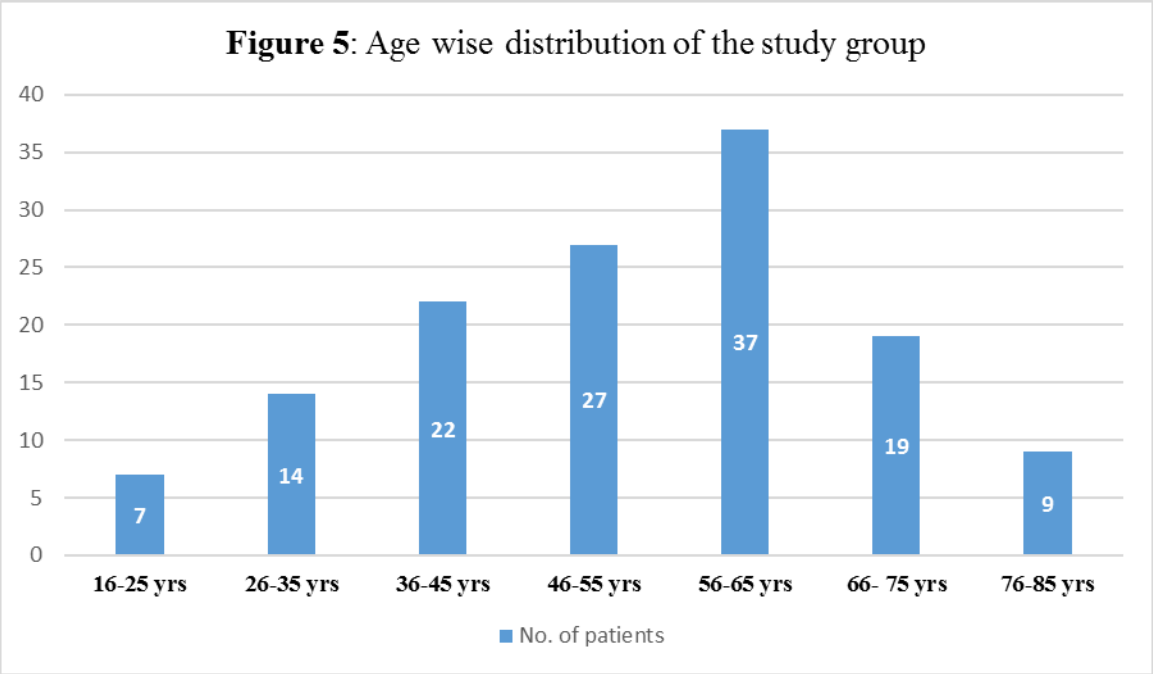


Figure 5: Age wise distribution of the study group

Sixty (44.44%) samples were from patients admitted in the intensive care units (ICU) and formed the majority of the study group. Patients admitted in the gastroenterology wards formed the second highest group with 30 (22.22%) patients developing antibiotic associated diarrhea as shown in Table 4.

Table 4: Ward wise distribution of the patients

WARD	NO. OF CASES	PERCENTAGE OF CASES
ICU	60	44.44%
Gastroenterology	30	22.22%
Neurology	9	6.67%
Semiprivate and Special wards	27	20%
General wards	3	2.22%
Oncology	3	2.22%
Nephrology	2	1.48%
Surgery	1	0.74%
Total	**135**	**100%**

Culture was performed on all the samples after enrichment in Robertson's cooked meat medium (Figure 6) and anaerobic environment was provided using the Anoxomat (Figure 7). preliminary identification of the organism was done on the basis of the colony morphology. *Clostridium difficile* grew as non-hemolytic, large, shiny, irregular grey white colonies, with typical horse stable odour on anaerobic blood agar after 48 hours of incubation (Figure 8). On CCFA, it produced flat, yellow, circular to irregular colonies, measuring

4 to 8mm after 48 hours of incubation (Figure 9). The colonies on CCFA produce yellow- green fluorescence under long UV light (Figure 10).

Gram staining showed large Gram positive rods with subterminal spores seen occasionally (Figure 11). All (100%) of the isolates were catalase negative, oxidase negative, esculin positive, lipase and lecithinase negative. The isolates fermented glucose, mannitol and fructose within 24 hrs (Figure 12 and Figure 13). All the isolates were sensitive to metronidazole.

Out of the 135 samples; 14 (10.37%) were positive for *C. difficile* by culture and 14 (10.37%) were positive by enzyme immunoassay (Figure 14) and 15 (11. 11%) were positive by PCR (Table 5). PCR was done on all cultured isolates and from the stool specimen of EIA positive samples, for tpi gene (Figure 15-17). This is a housekeeping gene for *C. difficile* which is species specific and forms a 230bp amplicon on gel electrophoresis. Out of 15 samples which were positive either by culture or EIA or both methods, all (100%) of the positive isolates and samples were positive for this gene.

Thirteen samples were positive by all three methods. Culture was considered as the gold standard and results by rapid membrane enzyme immunoassay were compared against it as shown in Table 6.

Table 5: Comparison of various methods for identification of *C. difficile*

Sample No.	Culture	EIA for GDH Ag	PCR for tpi gene (housekeeping gene)
1.	+	+	+
7.	+	+	+
11.	+	+	+
18.	+	+	+
26.	+	+	+
35.	+	+	+
45.	-	+	+
55.	+	+	+
61.	+	-	+
63.	+	+	+
73.	+	+	+
82.	+	+	+
104.	+	+	+
114.	+	+	+
123.	+	+	+
Total	**14**	**14**	**15**

Table 6: Comparative evaluation of rapid membrane enzyme immunoassay for GDH Ag with culture

CULTURE	EIA FOR GDH Ag		TOTAL	P VALUE (Pearson's Chi-squared test)
	POSITIVE	NEGATIVE		
POSITIVE	13	1	14	$p < 0.05$
NEGATIVE	1	120	121	
TOTAL	14	121	135	

Sensitivity of EIA= 13/14 x 100= 92.9%

Specificity of EIA= 120/121 x 100= 99.2%

Positive predictive value= 13/14 x 100= 92.9%

Negative predictive value= 120/121 x 100= 99.2%

Accuracy= 13+120/135 x 100= 98.5%

From Table 6, the EIA showed a sensitivity and positive predictive value of 92.9%. The specificity and negative predictive value was 99.2%. The accuracy of the rapid assay was 98.5%. Chi square test was performed which showed it to be statistically significant ($p < 0.05$). One of the samples was negative by EIA but was culture positive as well as positive for the tpi gene. One sample did not grow on culture, but was EIA positive and PCR from the stool sample was positive for the tpi gene and was considered as a true positive.

Of the 14 samples that were positive by EIA, 2(14.29%) were toxigenic. On the other hand, among the 15 samples that were PCR positive, 4(26.67%) of the samples were positive for toxigenic genes i.e., 3 were positive for both tcdA and tcdB and 1 was positive for only tcdA (Figure 18). None of the samples were positive for the binary toxins cdtA or cdtB (Table 7).

Table 7: Comparison of EIA and PCR for toxin positivity

Sample No.	**EIA for toxin**	**PCR for tcdA**	**PCR for tcdB**	**PCR for cdtA**	**PCR for cdtB**
1.	+	+	+	-	-
11.	+	+	+	-	-
26.	-	+	-	-	-
55.	-	+	+	-	-
Total	2	4	3	0	0

Demographic analysis of the patients showed that among the 15 samples that were positive by various methods, 13 (86.67%) belonged to male patients and 2 (13.33%) belonged to female patients (Fig. 19).

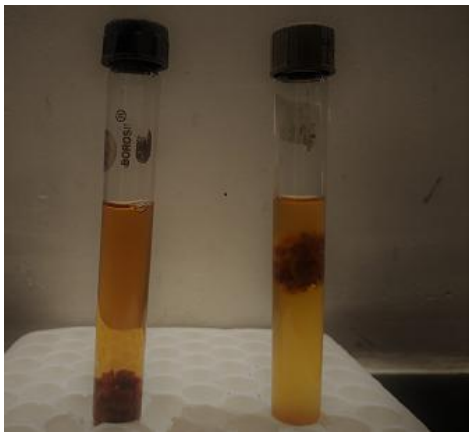


Figure 6: Sample inoculated in Robertson's cooked meat medium

Figure 7: Anoxomat

Figure 8: *C. difficile* on Anaerobic blood agar

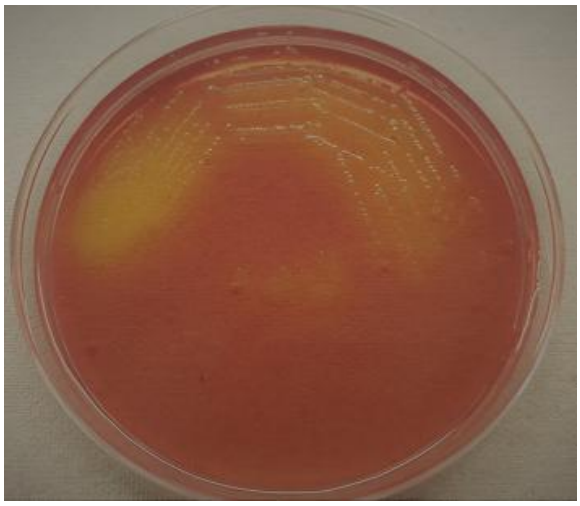


Figure 9: *C. difficile* on CCFA

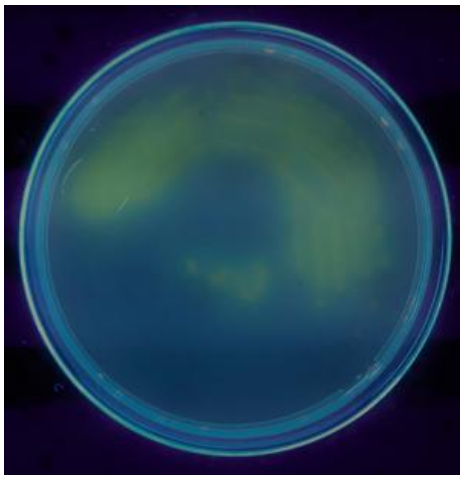


Figure 10: *C. difficile* colonies under long UV light

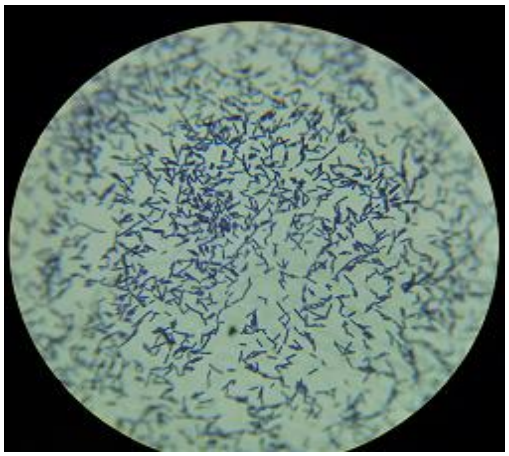


Figure 11: Gram stain appearance of *C. difficile*

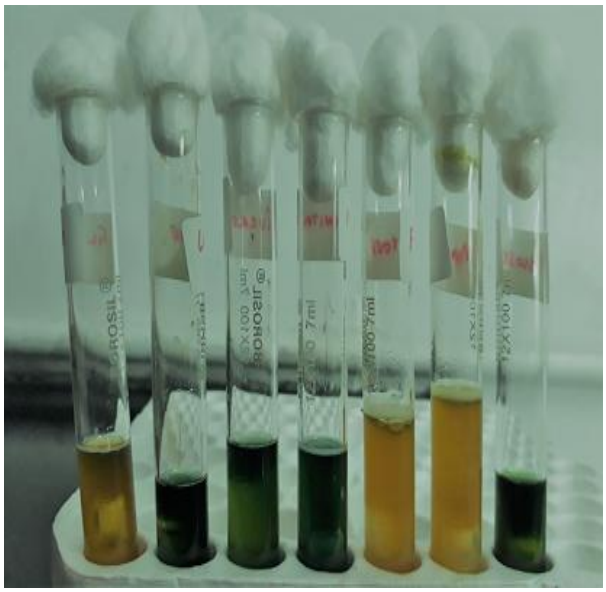


Figure 12: Carbohydrate fermentation (From left to right- Glu+,Lac-,Suc-, Mal-,Fru+,Man+,Xyl-)

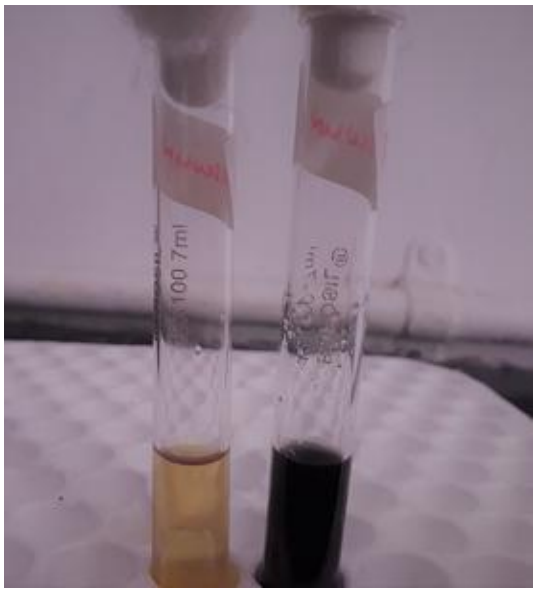


Figure 13: Esculin hydrolysis (Left- Control tube, Right- Positive sample)

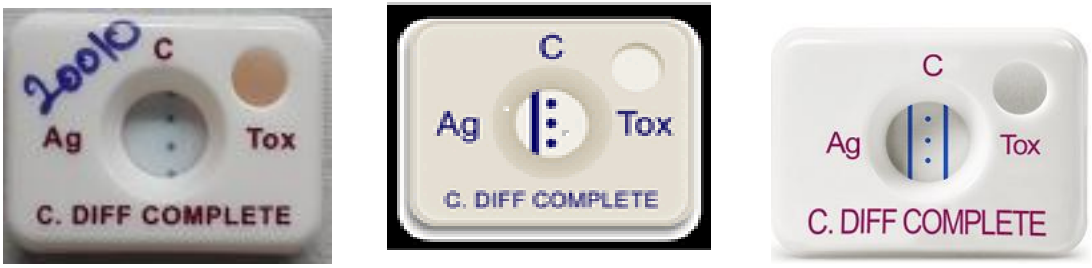


Figure 14: C. diff Quik chek Rapid Enzyme immunoassay

(From left to right- Negative sample, GDH Ag + sample, GDH+, Tox A/B+ sample)

Figure 15: Applied Biosystems Step One Real time PCR for

DNA amplification

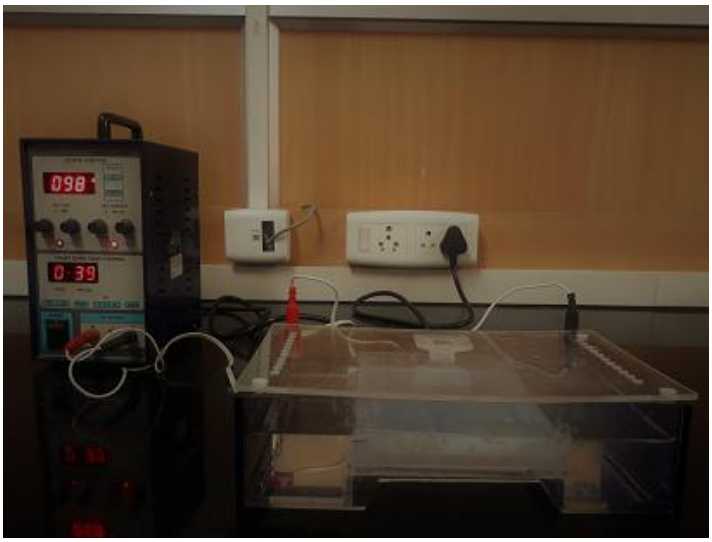


Figure 16: Gel electrophoresis

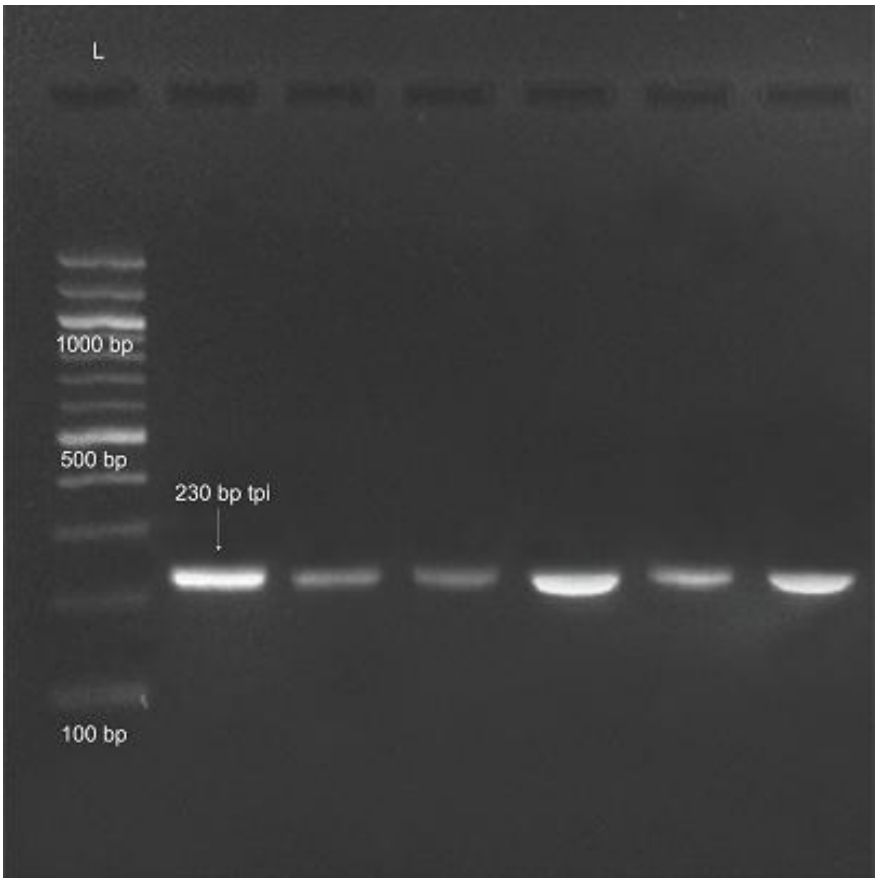


Figure 17: Gel electrophoresis picture showing tpi gene

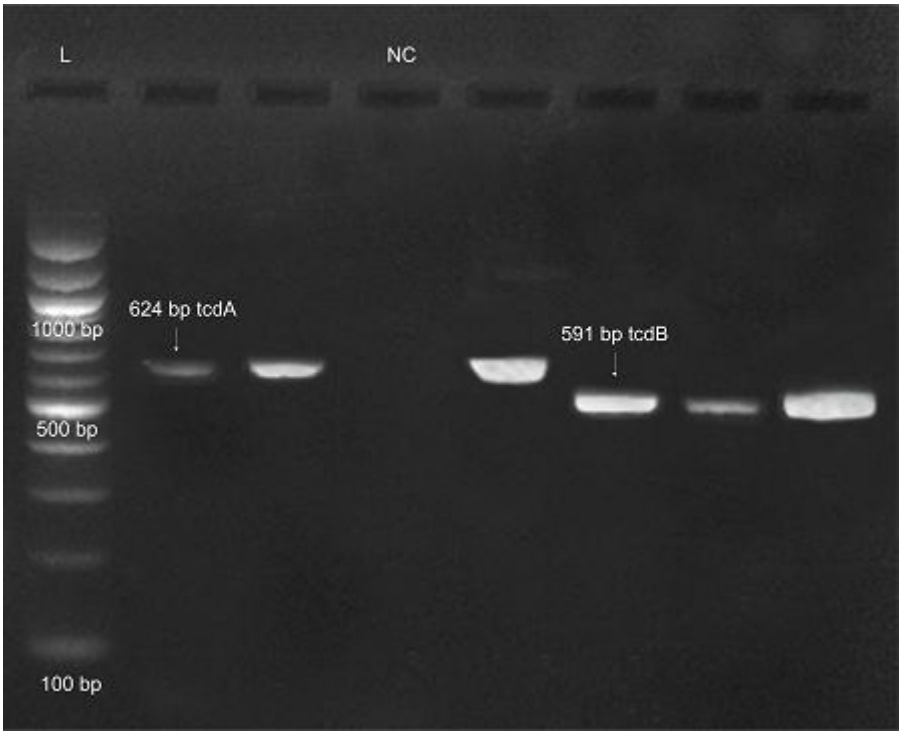


Figure 18: Gel electrophoresis picture showing tcd A and tcdB genes

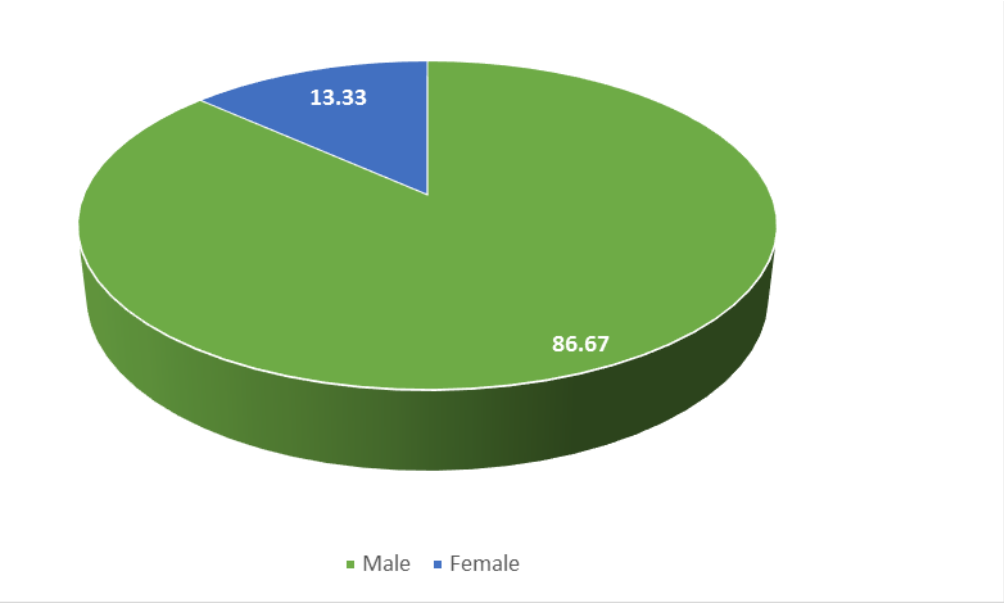


Figure 19: Sex distribution of the positive samples

The mean age of the positive cases was 45 years, but 6 (40%) of the positive cases occurred in patients between 56- 65years (Fig. 20).

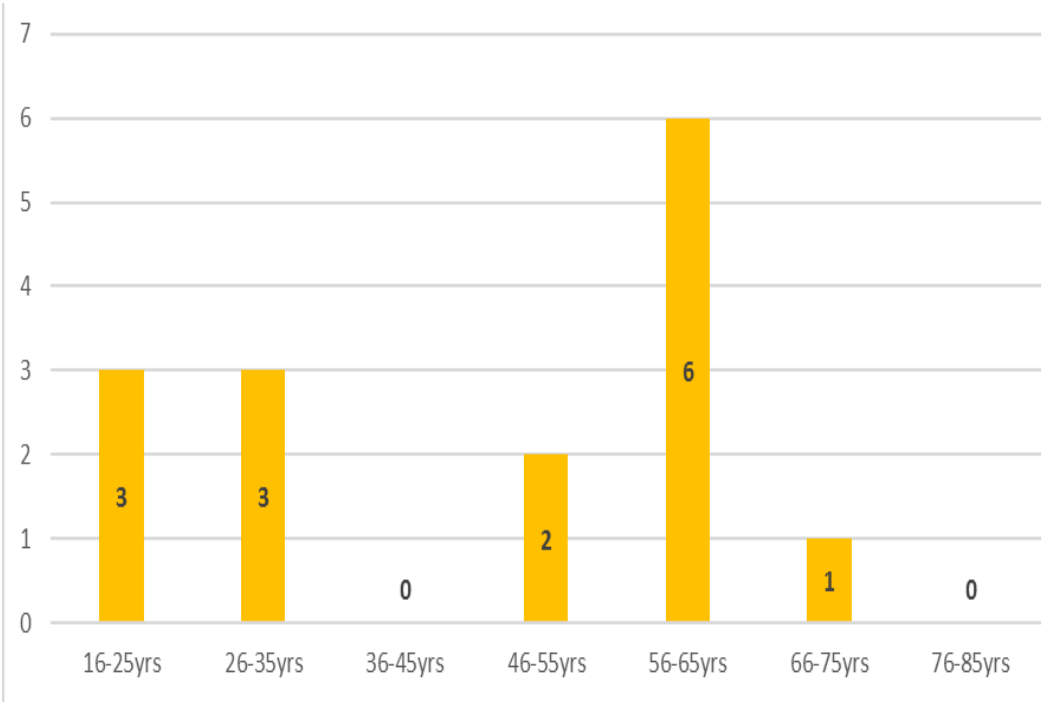


Figure 20: Age wise distribution of the positive samples

Eleven (66.67%) of the positive samples were from patients admitted in the ICU. The remaining were from patients admitted in Gastroenterology, Neurosurgery wards (Table 8).

Table 8: Ward wise distribution of positive cases

Ward	**No. of patients positive for *C. difficile***	**% of patients**
ICU	11	73.33%
Gastroenterology	2	13.33%
Neurosurgery	2	13.33%
Total	**15**	**100%**

The mean duration for colonization with *C. difficile* was 13.53 days in hospitalized patients. This was calculated by noting down the day of hospitalization and the time to positivity of the sample.

Seven (46.67%) of the positive samples were from patients who were diagnosed to have sepsis. Four (26.67%) of the positive patients were admitted for acute pancreatitis. Two (13.33%) of the positive samples were from immunocompromised patients. The other isolates were from patients admitted for other disorders such as cerebrovascular accident, pneumonia, etc. None of the patients admitted with ulcerative colitis showed growth of *C. difficile*.

In all 15(100%) patients, loose stools was the only symptoms. There was no associated blood in stools, fever, vomiting, abdominal pain or abdominal distension.

Most of the patients with *C. difficile* colonization had been treated with multiple antibiotics prior to the onset of diarrhea. Treatment with Piperacillin-tazobactum was noted in 6 (40%) patients and with Meropenem in 6 (40%) of the patients (Fig. 21), while 1 of the patients had received both antibiotics. One patient was on Vancomycin and although the sample was Ag positive by EIA and PCR positive for tpi gene, it did not grow on culture.

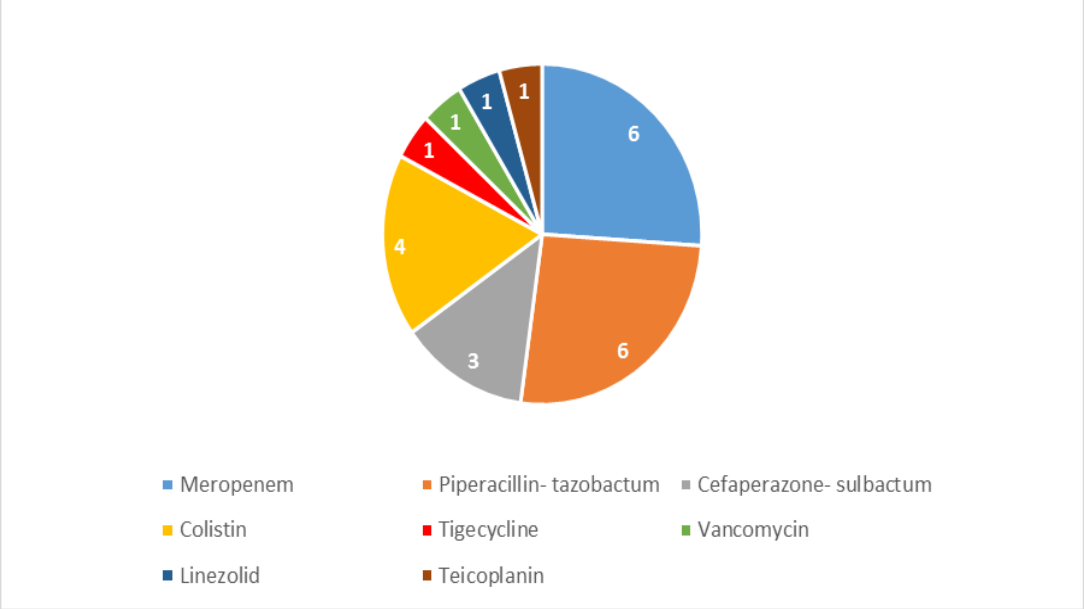


Figure 21: Antibiotic usage in patients with *C. difficile*

One of the patients was on immunosuppressive therapy with Cyclosporine following a liver transplant surgery. Thirteen (86.67%) of the patients had been on proton pump inhibitors prior to the development of diarrhea.

Following diagnosis, Metronidazole was the preferred drug with 11(73.33%) of the patients being treated with it exclusively or in combination with other agents (Fig. 22). One of the patients on treatment with Metronidazole for CDI continued to show growth three weeks after therapy and was started on Vancomycin. Three of the patients expired due to their underlying causes and the deaths were unrelated to CDI.

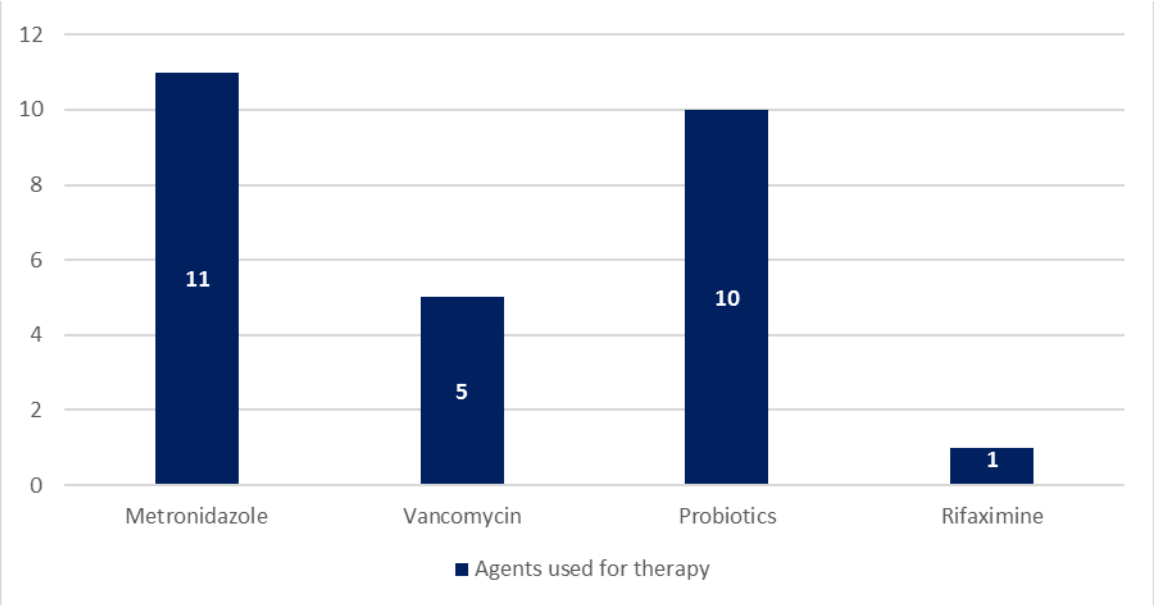


Figure 22: Agents used for therapy

DISCUSSION

The present study was aimed at determining the incidence of *Clostridium difficile* infection in hospitalized patients who develop diarrhea following initiation of antibiotics and to evaluate the usefulness of rapid membrane enzyme immunoassay in its diagnosis when compared to culture and PCR.

Diarrhea is a common adverse effect of antibiotic therapy with an incidence of 5-25% depending on the antibiotic used. This occurs due to a large scale alteration in the gut microbiota. Antibiotic associated diarrhea is considered clinically significant when the patient develops three or more loose stools per day. There are various infectious and non-infectious causes for the development of antibiotic associated diarrhea but frequently the causative agent cannot be determined. Among the infectious agents, *C. difficile* is the most commonly isolated pathogen. Other pathogens that are linked with AAD include *Staphylococcus aureus, Klebsiella oxytoca, Candida spp., Clostridium perfringens* and *Salmonella spp.* [60]

C. difficile is implicated in a wide spectrum of diseases called *C. difficile* infections (CDI) ranging from self-limiting antibiotic associated diarrhea to the more fatal pseudomembrane colitis. Although administration of antibiotics is the major risk factor for the development of the disease, there are other risk factors such as long term hospitalization, stay in intensive care units, immunosuppression, administration of gastric acid suppressants and

gastrointestinal surgical procedures. It is now recognized as a major health problem with significant medical and economic consequences. Therefore, prompt identification of these diseased patients is the need of the hour. [4- 6]

In this study, consecutive fecal samples from 135 patients who were clinically suspected to have antibiotic associated diarrhea due to *C. difficile* were processed. Culture was taken as the gold standard for the diagnosis of *C. difficile.* Fourteen (10.4%) of the samples grew the organism on culture. This was confirmed by the characteristic appearance of the colonies on anaerobic blood agar, the selective medium CCFA and by biochemical confirmation. IDSA guidelines also recommend usage of CCFA for isolation of *C. difficile.* [61] We found this medium to be highly selective for *C. difficile.* As reported earlier, addition of 0.1% sodium taurocholate to the medium is recommended especially while recovering the organism from stock cultures as it helps in the germination of the spores. [62] Among the carbohydrate fermentation tests, fermentation of mannitol is most important in the identification of *C. difficile* as other clostridial species do not ferment mannitol. [63]

The incidence of *C. difficile* in our study is lower than that reported by foreign studies with a majority of studies reporting an incidence between 15-25%. [43] Studies in India have shown varied results. Our findings concur with the findings of Ingle *et al.*, Dutta *et al.* and Niyogi and Bhattacharya *et al.* who have reported an incidence of 7-11% in hospitalized patients who developed acute diarrhea. A study conducted in 2011 by Vaishnavi *et al.* showed a higher incidence of 30% in hospitalized patients receiving single or multiple

antibiotics. [4, 64] The decrease in incidence over the past decade has been noted in a few studies. This might be due to expanded prevention and control efforts, implementation of antibiotic policies in hospitals and changes in the prevalence of the epidemic strains. [43]

Majority (70.4%) of the samples in our study belonged to male patients. Although age > 65 years has been classically described as a risk factor for the development of AAD, the current study showed a higher incidence (47.4%) in the middle aged group (46-65 years) and a lower incidence (20.7%) among the geriatric group (66- 85 years). Similar age and sex preponderance was noted among the positive isolates as well. Several other studies have shown varying results with a few reporting increased incidence in AAD among male patients and few others among female patients. [65- 67] Hence gender of the patient might not be a significant risk factor in the development of AAD. Only few studies on adult AAD have provided age or gender data with two studies reporting the mean age as 49-72 years. [68]

As reported earlier, the majority (44.4%) of our study group was from patients admitted in the intensive care units with 18.3% of the samples from ICU being positive for *C. difficile*. Other studies reported a prevalence between 15-38% from patients in critical care units. [69] Patients in intensive care units are usually on enteral feeding and this is the most common cause of diarrhea in this population. Other factors contributing to the development of diarrhea include hypoalbuminemia, intestinal ischemia and medication induced diarrhea. CDI is also common in this population as these patients are exposed

to multiple broad spectrum antibiotics, corticosteroids, proton pump inhibitors and also suffer from underlying comorbid conditions such as renal insufficiency, diabetes, gastrointestinal disease and immunodeficiency. [70, 71]

The mean duration for hospitalization in our study, prior to diagnosis of CDI, was 13.53 days. Similar results were seen in a study by Dávila *et al.* who reported an average duration of 13.6 days prior to CDI diagnosis while Chalmers *et al.* reported an average of 18 days.[72, 73] As this is a nosocomial infection, there is a higher risk of acquiring the infection following prolonged hospitalization due to persistence of spores which are present in the hospital environment.

Due to the associated morbidity and mortality, early diagnosis of CDI is crucial as it responds well to therapy. There is no consensus as to what the gold standard is as early studies considered the cell culture cytotoxicity neutralisation assay as the standard while recent studies consider toxigenic culture as the gold standard. [12, 41] However, both these methods are time consuming and not feasible in resource limited settings. In such a scenario, rapid diagnostic tests are the need of the hour. In our study, we used the C. diff Quik Chek complete which currently is the only lateral flow enzyme immunoassay that detects GDH Ag as well as Toxin A/B simultaneously. Some investigators have found this test to have better sensitivities when compared to the two step testing algorithm where GDH is first detected followed by testing separately for the toxin in the GDH positive samples. Sharp *et al.* reported a sensitivity and specificity of 97.6% and 94.6% respectively with the GDH

component of the test while the toxin component showed a sensitivity and specificity of 61.9% and 99.2% respectively. [55] However, the disadvantage is that it is comparatively more expensive. *C. difficile* strains produce the cell wall associated enzyme glutamate dehydrogenase (GDH) antigen which is highly conserved among the various ribotypes and is independent from the pathogenicity locus. [74] Rapid tests utilize its detection as a screening test for diagnosis of CDI.

Comparative analysis of the assay for GDH Ag in our study revealed a sensitivity and specificity of 92.9% and 99.2% respectively when compared to culture and an accuracy of 98.5% (p value < 0.05). Swindell *et al.* reported a sensitivity of 100% and specificity of 97% on comparison with toxigenic culture. Other studies have also reported similar findings. [55, 75]. This makes the rapid assay an effective screening test for diagnosis of CDI.

PCR is another useful diagnostic tool for diagnosis of CDI and detection of toxin genes. Newer developments are available such as the nucleic acid amplification techniques (NAAT) which are highly sensitive and specific but are not cost effective. In the present study, all samples which were positive by culture or rapid assay were further confirmed by testing for the tpi gene by conventional PCR. The triose phosphate isomerase gene is a housekeeping gene which is species specific and considered to be more discriminatory than 16s ribosomal DNA for the identification of the species within the genus *Clostridium.*[56] All the culture positive isolates were positive for this gene. One of the samples was negative by culture and positive by the rapid assay for

GDH. However, direct PCR from the stool sample in this patient was also positive for the tpi gene. Hence, it can be considered as a true positive test. The patient was already on treatment with Vancomycin when the sample was tested and this might be why it failed to grow on culture.

Culture and positive GDH Ag however only indicate colonization in the gut by *C. difficile.* Toxin detection needs to be done to confirm CDI. Toxin A and Toxin B are the major toxins which are encoded by tcdA and tcdB respectively. Toxin A is an enterotoxin and Toxin B is a cytotoxin. Disease manifestation and complications are mainly due to the action of these toxins. In addition to this, some strains possess a binary toxin encoded by cdtA and cdtB genes. This can also act as a virulence factor and is associated with certain epidemic strains.

Although the rapid assay is highly specific (100%) for detection of toxin, studies have reported a low sensitivity, ranging from 61-78%. [(76)] Two out of 14 samples (14.29%) were positive for toxin by the rapid assay in our study. Toxin production was confirmed by detection of the toxigenic genes tcdA, tcdB, cdtA and cdtB by PCR. It was not possible to test all the samples for the presence of these toxigenic genes due to economic constraints and only the samples which were positive by culture or rapid assay were tested. 4 out of 15 samples (26.67%) were positive for toxin genes by PCR. Both the samples which tested positive for toxin by rapid assay were positive for tcdA and tcdB by PCR. In addition to this, two samples which were negative by rapid assay were positive for toxins- one was positive was for both tcdA and tcdB, while

the other was positive for only tcdA. A significant difference was thus noted in the detection of toxins by rapid assay as compared to PCR. None of the positive samples showed the presence of the binary toxin genes.

A similar study conducted by Vaishnavi *et al.* in Chandigarh showed higher rates of toxigenic isolates. 174 culture positive isolates were tested by PCR and 69.5% of these isolates were found to be toxigenic in their study. 9.2% of these isolates were also positive for the binary toxin genes. No other study in India has reported the presence of the binary toxin genes. [77] Lower rates were reported by Chaudhry *et al.* in a study conducted in Delhi where 7.1% of stool samples from patients suspected to have CDAD were toxigenic. But by and large, *C. difficile* is an underdiagnosed condition in India with not many studies being done on it.

All our patients with positive culture or toxin assay had loose stools as the only symptom. Majority of the patients were on Meropenem and Piperacillin- tazobactum (40%) prior to onset of the disease and later developed diarrhea. Yeung *et al.* reported an incidence of 3.5 and 4.9% following exposure to these antibiotics. [78] None of our patients with positive results were on fluoroquinolones or clindamycin which is generally implicated in the development of CDAD. However, it is difficult to pinpoint which antibiotics are responsible for the disease due to other confounding factors such as usage of proton pump inhibitors. A large observational study by Loo *et al.* showed a strong association between proton pump inhibitor usage and the development of disease. [79] Intake of proton pump inhibitors was noted in 86.67% of the

patients with positive cultures in our study. This is a risk factor for the development of disease as the vegetative forms of the bacteria are sensitive to the presence of gastric acid in the stomach.

Following diagnosis, 73.3% were treated with Metronidazole and supportive treatment with probiotics while others were treated with Vancomycin. One patient who tested positive for toxigenic *C. difficile* continued to have persistent diarrhea despite treatment with Metronidazole and responded to treatment with Vancomycin. None of the patients developed further complications.

This study highlights the importance of identification of *C. difficile* in hospitalized patients with antibiotic associated diarrhea. In a developing country like India, this condition is frequently underdiagnosed. Failure to diagnose can result in further complications in the patient and can even lead to mortality. Our study also evaluated the efficacy of rapid diagnostic assays when compared to culture and PCR. Understanding the incidence, will help us in implementing better preventive measures and stringent antibiotic policies in hospitals.

SUMMARY

- *C. difficile* is the most common infectious agent that is implicated in hospitalized patients with antibiotic associated diarrhea. Hence, this study was conducted to estimate the incidence of *C. difficile* associated diarrhea, evaluate the risk factors and to compare rapid enzyme immunoassays with culture and PCR.
- Stool samples from 135 hospitalised patients with antibiotic associated diarrhea and who were suspected to have *C. difficile* infection were collected.
- Most of the samples were from male patients (70.4%) and patients who were in the middle age group between 46-65years (47.4%).
- Majority of samples (44.4%) were from patients in intensive care units
- *C. difficile* was isolated in 10.37% of the samples by culture on anaerobic blood agar, the selective medium cycloserine- cefoxitin fructose agar and identification was confirmed by biochemical tests. This was considered as the gold standard.
- Samples were also tested by the rapid enzyme immunoassay which detects glutamate dehydrogenase (GDH) Ag and toxin A/ B simultaneously. Rapid assay for GDH Ag showed a sensitivity and specificity of 92.9% and 99.2% respectively ($p < 0.05$) when compared to culture. Two of these samples were toxin positive.
- Culture positives and samples which were positive by the rapid assay were further tested by PCR for the housekeeping gene tpi and all were

found to be positive. One sample which was culture negative but positive by the immunoassay was also positive by PCR and can be considered a true positive.

- PCR confirmed the presence of toxigenic genes tcdA and tcdB in the two samples which were positive by rapid assay. Two samples which were positive for GDH Ag but toxin negative by rapid assay were positive for toxin genes by PCR. One was $tcdA^{+}tcdB^{+}$ and the other was positive for only tcdA.
- None of the samples were positive for the binary toxin genes cdtA and cdtB.
- Mean duration for hospitalization was 13.53 days prior to diagnosis of CDI.
- Majority (40%) of patients were on Meropenem and Piperacillin-tazobactum prior to diagnosis.
- Intake of proton pump inhibitors was noted in 86.67% of patients which is a significant risk factor.
- Patients responded well to therapy with Metronidazole and Vancomycin and none developed complications. One patient continued to have persistent diarrhea and sample was culture positive despite treatment with Metronidazole but responded to therapy with Vancomycin.

CONCLUSION

Clostridium difficile is the most common pathogen implicated in nosocomial diarrhea. Early diagnosis of this condition is necessary as it can lead to considerable morbidity and mortality. Culture of faecal specimens in hospitalized patients with antibiotic associated diarrhea in our study revealed an incidence of 10.4%. Although culture is the gold standard for diagnosis, it is time consuming and not feasible in resource limited settings. 26.7% of the cultured isolates were identified as toxigenic strains by PCR. PCR is highly sensitive and specific but expensive. In such a scenario, rapid assays can serve as effective screening tests for diagnosis of the disease with our results showing a sensitivity and specificity of 92.9% and 99.2% respectively. This can be followed by testing for toxin production. A reduction in the incidence can be brought about in hospitals by simple measures such as handwashing, contact precautions and formulating antibiotic policies to prevent indiscriminate use of antibiotics.

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Appendix

ABBREVIATIONS

AAD- Antibiotic associated diarrhea

CCCNA- Cell culture cytotoxicity neutralization assay

CCFA- Cycloserine cefoxitin fructose agar

CDAD- *Clostridium difficile* associated diarrhea

CDC- Centre for Disease Control and Prevention

CDI- *Clostridium difficile* infection

EIA- Enzyme immunoassay

FMT- Fecal microbiota transplantation

GDH- Glutamate dehydrogenase

IDSA- Infectious Diseases Society of America

MIC- Minimum inhibitory concentration

NAAT- Nucleic acid amplification techniques

PCR- Polymerase chain reaction

PMC- Pseudomembranous colitis

RCM- Robertson's cooked meat broth

TC- Toxigenic culture

PREPARATION OF REAGENTS AND MEDIA

1) GRAM STAIN

Crystal violet:

Crystal violet	1.0gm
5% sodium bicarbonate	1.0ml
Distilled water	99ml

Grams iodine:

Iodine crystal	2.0gm
Sodium hydroxide	10.0ml
Distilled water	90.0ml

Acetone- 100%

Dilute carbol fuschin- 1 in 10 dilution of strong carbol fuschin

2) THIOGLYCOLLATE BROTH

Enriched thioglycollate broth (HiMedia)	3.1gm
Distilled water	100ml

The medium is added to distilled water and sterilized by autoclaving at 121°C for 15 minutes at 15lbs.

3) ROBERTSON'S COOKED MEAT BROTH

Cooked meat medium (HiMedia)	11.54gm
Thioglycollate broth	100ml

The dehydrated meat particles are added to thioglycollate broth and allowed to stand until all the particles are thoroughly wetted. This is dispensed in screw capped tubes and autoclaved at 121°C for 15 minutes at 15lbs.

4) ANAEROBIC BLOOD AGAR

Anaerobic blood agar base (HiMedia)	44gm
Distilled water	1000ml

The medium is completely dissolved in distilled water and autoclaved at 121°C for 15minutes at 15lbs. It is cooled to 45- 50°C and 1% neomycin solution, 1 vial of Vitamin K supplement (HiMedia) and 5% defibrinated sheep blood is added. The medium is mixed well and poured into sterile petri plates.

5) CYCLOSERINE- CEFOXITIN FRUCTOSE AGAR

Clostridium difficile agar base	34.55gm
Distilled water	500ml

The medium is completely dissolved in distilled water and autoclaved at 121°C for 15minutes at 15lbs. It is cooled to 45- 50°C and 1 vial of Clostridium difficile supplement (HiMedia) along with 1% neutral red solution is added. The medium is mixed well and poured into sterile petri plates.

6) CARBOHYDRATE FERMENTATION MEDIUM

Carbohydrate	1gm
Thioglycollate broth	100ml

The carbohydrate is added to thioglycollate broth and sterilised by autoclaving at 115°C for 10 minutes.

7) ESCULIN HYDROLYSIS BROTH

Aesculin	0.5gm
Thioglycollate broth	100ml

Aesculin powder is added to the broth and sterilised by autoclaving at 115°C for 10 minutes.

Annexure

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

January 2, 2017

To
Dr Sneha Mary Kurian
Postgraduate
Department of Microbiology
Guide: Dr Marina Thomas
PSG IMS & R
Coimbatore

The Institutional Human Ethics Committee, PSG IMS & R, Coimbatore - 4, has reviewed your proposal on 2nd January 2017 in its expedited review meeting held at IHEC Secretariat, PSG IMS&R, between 10.00 am and 11.00 am, and discussed your request to renew the approval for the study entitled:

"Isolation and characterization of Clostridium difficile and toxin detection from faecal specimens in patients with antibiotic associated diarrhea in a tertiary care centre"

The following documents were received for review:

1. Request for renewal dated 27.12.2016
2. Status Report

After due consideration, the Committee has decided to renew the approval for the above study.

The members who attended the meeting held on at which your proposal was discussed, are listed below:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr R Nandakumar (Chairperson, IHEC)	BA., BL	Legal Expert	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr S Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr Sudha Ramalingam	MD	Epidemiologist, Ethicist Alt. member-Secretary	Female	Yes	Yes
5	Dr D Vijaya	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The approval is valid for one year (29.12.2016 to 28.12.2017).

This Ethics Committee is organized and operates according to Good Clinical Practice and Schedule Y requirements.

Non-adherence to the Standard Operating Procedures (SOP) of the Institutional Human Ethics Committee (IHEC) and national and international ethical guidelines shall result in withdrawal of approval (suspension or termination of the study). SOP will be revised from time to time and revisions are applicable prospectively to ongoing studies approved prior to such revisions.

Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Yours truly,

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr S Bhuvaneshwari
Member – Secretary
Institutional Human Ethics Committee
Proposal No. 15/405

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

To
Dr Sneha May Kurian
Postgraduate
Department of Microbiology
Guide: Dr Marina Thomas
PSG IMS & R
Coimbatore

Ref: Project No.15/405

Date: December 29, 2015

Dear Dr Sneha May Kurian,

Institutional Human Ethics Committee, PSG IMS&R reviewed and discussed your application dated 19.12.2015 to conduct the research study entitled *"Isolation and characterization of Clostridium difficile and toxin detection from faecal specimens in patients with antibiotic associated diarrhea in a tertiary care centre"* during the IHEC meeting held on 24.12.2015.

The following documents were reviewed and approved:

1. Project Submission form
2. Study protocol (Version 1 dated 19.12.2015)
3. Confidentiality statement
4. Application for waiver of consent
5. Data collection tool (Version 1 dated 19.12.2015)
6. Current CVs of Principal investigator, Co-investigators
7. Budget

The following members of the Institutional Human Ethics Committee (IHEC) were present at the meeting held on 24.12.2015 at IHEC Secretariat, PSG IMS & R between 10.00 am and 11.00 am:

Sl. No.	Name of the Member of IHEC	Qualification	Area of Expertise	Gender	Affiliation to the Institution Yes/No	Present at the meeting Yes/No
1	Mr. R. Nandakumar	BA., BL	Legal Expert, Chairperson	Male	No	Yes
2	Dr. S. Bhuvaneshwari (Member-Secretary, IHEC)	MD	Clinical Pharmacology	Female	Yes	Yes
3	Dr. S. Shanthakumari	MD	Pathology, Ethicist	Female	Yes	Yes
4	Dr D Vijaya	M Sc., Ph D	Basic Medical Sciences (Biochemistry)	Female	Yes	Yes

The study is approved in its presented form. The decision was arrived at through consensus. Neither PI nor any of proposed study team members were present during the decision making of the IHEC. The IHEC functions in accordance with the ICH-GCP/ICMR/Schedule Y guidelines. The approval is valid until one year from the date of sanction. You may make a written request for renewal / extension of the validity, along with the submission of status report as decided by the IHEC.

PSG Institute of Medical Sciences & Research
Institutional Human Ethics Committee

Recognized by The Strategic Initiative for Developing Capacity in Ethical Review (SIDCER)
POST BOX NO. 1674, PEELAMEDU, COIMBATORE 641 004, TAMIL NADU, INDIA
Phone : 91 422 - 2598822, 2570170, Fax : 91 422 - 2594400, Email : ihec@psgimsr.ac.in

Following points must be noted:

1. IHEC should be informed of the date of initiation of the study
2. Status report of the study should be submitted to the IHEC every 12 months
3. PI and other investigators should co-operate fully with IHEC, who will monitor the trial from time to time
4. At the time of PI's retirement/intention to leave the institute, study responsibility should be transferred to a colleague after obtaining clearance from HOD, Status report, including accounts details should be submitted to IHEC and extramural sponsors
5. In case of any new information or any SAE, which could affect any study, must be informed to IHEC and sponsors. The PI should report SAEs occurred for IHEC approved studies within 7 days of the occurrence of the SAE. If the SAE is 'Death', the IHEC Secretariat will receive the SAE reporting form within 24 hours of the occurrence
6. In the event of any protocol amendments, IHEC must be informed and the amendments should be highlighted in clear terms as follows:
   a. The exact alteration/amendment should be specified and indicated where the amendment occurred in the original project. (Page no. Clause no. etc.)
   b. Alteration in the budgetary status should be clearly indicated and the revised budget form should be submitted
   c. If the amendments require a change in the consent form, the copy of revised Consent Form should be submitted to Ethics Committee for approval
   d. If the amendment demands a re-look at the toxicity or side effects to patients, the same should be documented
   e. If there are any amendments in the trial design, these must be incorporated in the protocol, and other study documents. These revised documents should be submitted for approval of the IHEC and only then can they be implemented
   f. Any deviation-Violation/waiver in the protocol must be informed to the IHEC within the stipulated period for review
7. Final report along with summary of findings and presentations/publications if any on closure of the study should be submitted to IHEC

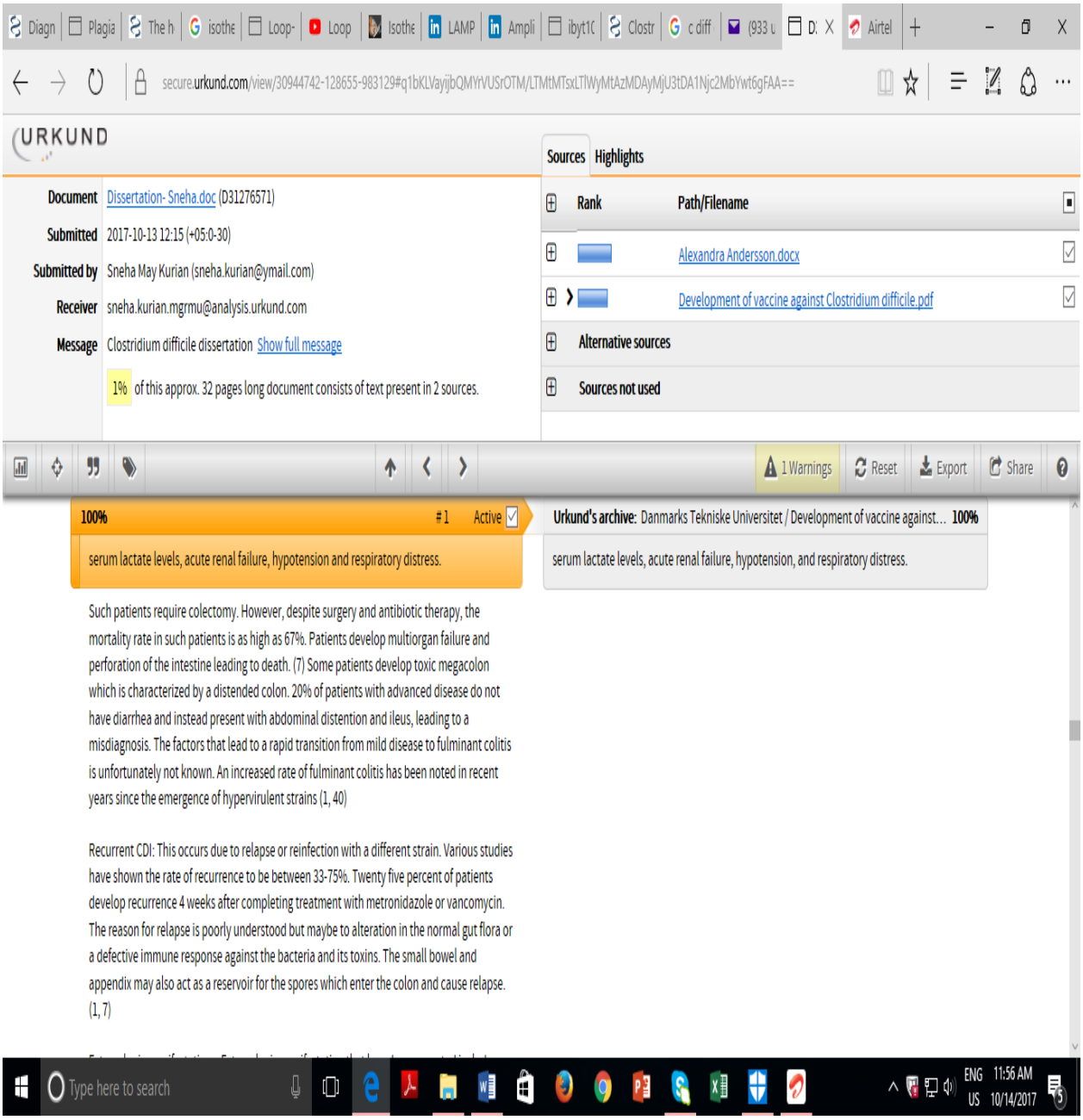
Kindly note this approval is subject to ratification in the forthcoming full board review meeting of the IHEC.

Thanking You,

Yours Sincerely,

SECRETARY
PSG IMS&R
COIMBATORE-641004.
INSTITUTIONAL HUMAN ETHICS COMMITTEE

Dr Sudha Ramalingam
Alternate Member - Secretary
Institutional Human Ethics Committee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Sources
Highlights
Document Dissertation- Sneha.doc (D31276571)
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Such patients require colectomy. However, despite surgery and antibiotic therapy, the mortality rate in such patients is as high as 67%. Patients develop multiorgan failure and perforation of the intestine leading to death. (7) Some patients develop toxic megacolon which is characterized by a distended colon. 20% of patients with advanced disease do not have diarrhea and instead present with abdominal distention and ileus, leading to a misdiagnosis. The factors that lead to a rapid transition from mild disease to fulminant colitis is unfortunately not known. An increased rate of fulminant colitis has been noted in recent years since the emergence of hypervirulent strains (1, 40)
Recurrent CDI: This occurs due to relapse or reinfection with a different strain. Various studies have shown the rate of recurrence to be between 33-75%. Twenty five percent of patients develop recurrence 4 weeks after completing treatment with metronidazole or vancomycin. The reason for relapse is poorly understood but maybe to alteration in the normal gut flora or a defective immune response against the bacteria and its toxins. The small bowel and appendix may also act as a reservoir for the spores which enter the colon and cause relapse. (1, 7)
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Urkund Analysis Result

Analysed Document: Dissertation- Sneha.doc (D31276571)
Submitted: 10/13/2017 8:45:00 AM
Submitted By: sneha.kurian@ymail.com
Significance: 1 %

Sources included in the report:

Alexandra Andersson.docx (D6068348)
Development of vaccine against Clostridium difficile.pdf (D21527804)

Instances where selected sources appear:

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